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PATENT

Attorney Docket No.: PRMG-04578

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE


Box Patent Application
 Assistant Commissioner For Patents
 Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of Michael R. Slater, Fen Huang and James R. Hartnett for **Thermophilic DNA Polymerases From *Thermotoga Neapolitana***.

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the U.S. Postal Service on this date **August 18, 2000** in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number **EL 541 885 870 US** addressed to: **Box Patent Application**, Assistant Commissioner For Patents, Washington, D.C. 20231.


 Mary Ellen Waite

1. **Type Of Application**

This new application is for a(n)

- ☒ Original (nonprovisional)
☒ Continuation.

2. **Benefit Of Prior U.S. Application(s)** (35 U.S.C. §§ 119(e), 120, or 121)

- ☒ The new application being transmitted claims the benefit of prior U.S. application(s) and enclosed are **ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.**

3. **Papers Enclosed That Are Required For Filing Date Under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application**

177 Pages of Specification
 3 Pages of Claims
 1 Page of Abstract
 6 Sheets of Informal Drawings

4. **Declaration**

- ☒ Enclosed
☒ Executed by inventors.

5. **Inventorship Statement**

The inventorship for all the claims in this application is:

- ☒ the same

6. **Language**

- ☒ English

7. **Fee Calculation (37 C.F.R. § 1.16)**

- ☒ Regular application

CLAIMS AS FILED

Number Filed	Number Extra	Rate	Basic Fee - \$760.00 (37 C.F.R. § 1.16(a))
Total Claims (37 C.F.R. § 1.16(c))	26 - 20 =	6 × \$18.00 =	\$108.00
Independent Claims (37 C.F.R. § 1.16(b))	13 - 3 =	10 × \$78.00 =	\$780.00
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))	+ \$260.00 =		\$0.00
Filing Fee Calculation			\$1648.00

8. **Fee Payment Being Made At This Time**

☒ Enclosed

☒ basic filing fee

\$1648.00

Total Fees Enclosed

\$1648.00

9. **Method of Payment of Fees**

☒ Check in the amount of \$1648.00

10. **Authorization To Charge Additional Fees and Credit Overpayment**

☒ The Commissioner is hereby authorized to charge payment of any fees associated with this communication or credit any overpayment to Deposit Account No.: 08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.


11. **Power of Attorney by Assignee**

☒ Enclosed (unsigned)

12. **Return Receipt Postcard**

☒ Enclosed

Dated: August 18, 2000


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☒ **Incorporation By Reference Of Added Pages**

☒ Plus Added Pages For New Application Transmittal Where Benefit Of Prior U.S. Application(s) Claimed

Number of pages added 1

ADDED PAGES FOR APPLICATION TRANSMITTAL
WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

13. Relate Back

A. 35 U.S.C. § 119(e)

B. 35 U.S.C. §§ 120, 121 and 365(c)

☒ Amend the Specification by inserting before the first line the sentence. "This is a Continuation of copending application(s) 09/385,986 filed on 08/30/99 which is a continuation of U.S. Patent Application Serial No. 08/484,661 now U.S. Patent No. 6,001,656."

14. Further Inventorship Statement Where Benefit Of Prior Application(s) Claimed

a. This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

☒ the same.

b. This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

☒ the same.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Slater, *et al.*

Serial No.:

Group No.:

Filed:

Examiner:

Entitled:

Thermophilic DNA Polymerases From *Thermotoga Neapolitana***PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents

Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence is being deposited with the U.S. Postal Service on this date **August 18, 2000** in an envelope as "Express Mail Post Office to Addressee: Mailing Label No. **EL 541 885 870** US addressed to: **Box Patent Application**, Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: August 18, 2000

By:


Mary Ellen Waite

Sir:

The Applicants have copied claims 29, 30, 32, 33, 34, 35, 37, and 38 from U.S. Patent No. 5,939,301, ("301") in order to preserve the right to provoke an interference proceeding with the patentees of the '301 patent. The Applicants are currently investigating the possibility of pursuing such interference proceeding. Claims 29, 30, 32, 33, 34, 35, 37, and 38 of the '301 patent correspond, respectively, to the Applicants' Claims 40, 41, 42, 43, 44, 45, 46, and 47.

Prior to an Examination on the merits of the above-identified Application, please amend the Application as follows:

IN THE SPECIFICATION

Please amend the Specification as follows:

After the Title of the invention, but before the field of the invention, please add --The present application is a Continuation of U.S. Patent Application Serial No. 09/385,986, which

is a Continuation of U.S. Patent Application Serial No. 08/484,661, now U.S. Patent No. 6,001,645.--.

IN THE CLAIMS:

Please cancel Claims 1-21.

Please add the following Claims:

22. A composition comprising a purified non-naturally-occurring DNA polymerase, or fragments thereof, capable of DNA synthetic activity, said polymerase derived from *Thermotoga neapolitana*.

23. A composition comprising a mutant DNA polymerase, said mutant polymerase derived from a *Thermotoga neapolitana* DNA polymerase.

24. The composition of Claim 23, wherein said mutant DNA polymerase comprises a mutation that reduces a 3'-5' exonuclease activity of said DNA polymerase.

25. The composition of Claim 23, wherein said mutant DNA polymerase comprises a mutation that reduces a 5'-3' exonuclease activity of said DNA polymerase.

26. The composition of Claim 23, wherein said mutant DNA polymerase comprises a mutation resulting in said DNA polymerase having reduced discrimination against dideoxynucleotides.

27. The mutant DNA polymerase of Claim 23, wherein said mutant DNA polymerase comprises one or more amino acid substitutions.

28. The mutant DNA polymerase of Claim 23, wherein said mutant DNA polymerase comprises one or more amino acid deletions.

29. The composition of Claim 23, wherein said mutant polymerase is devoid of an N-terminal 5'-3' exonuclease domain.

30. The composition of Claim 23, wherein said mutant polymerase is devoid of the 283 N-terminal amino acids of native *Thermotoga neapolitana* DNA polymerase.

31. A composition comprising an isolated nucleic acid encoding a mutant *Thermotoga neapolitana* DNA polymerase.

32. The composition of Claim 31, wherein said mutant DNA polymerase comprises a mutation that reduces a 3'-5' exonuclease activity of said DNA polymerase.

33. The composition of Claim 31, wherein said mutant DNA polymerase comprises a mutation that reduces a 5'-3' exonuclease activity of said DNA polymerase.

34. The composition of Claim 31, wherein said mutant DNA polymerase comprises a mutation resulting in said DNA polymerase having reduced discrimination against dideoxynucleotides.

35. The composition of Claim 31, wherein said DNA molecule is selected from the group consisting of pM284, pD323E, and pD323,389A.

36. The composition of Claim 31, wherein said DNA molecule further comprises expression control elements.

37. The composition of Claim 36, wherein said expression control elements comprise an inducible promoter.

38. A method of producing a mutant *Thermotoga neapolitana* DNA polymerase, said method comprising:

- (a) culturing a cellular host cell comprising a gene encoding a mutant *Thermotoga neapolitana* DNA polymerase;
- (b) expressing said gene; and
- (c) isolating said mutant *Thermotoga neapolitana* DNA polymerase from said host cell.

39. The method of Claim 38, wherein said host is *E. coli*.

40. A mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said polymerase, wherein said mutation is in the 3'-5' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

41. An isolated DNA molecule comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said polymerase, wherein said mutation is in the 3'-5' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

42. A recombinant host cell comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said polymerase, wherein said mutation is in the 3'-5' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

43. A method of producing a mutant *Thermotoga neapolitana* DNA polymerase, said method comprising:

- (a) culturing a host cell comprising a gene encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said polymerase, wherein said mutation is in the 3'-5' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase;

- (b) expressing said gene; and
- (c) isolating said mutant *Thermotoga neapolitana* DNA polymerase from said host cell.

44. A mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said polymerase, wherein said mutation is in the 5'-3' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

45. An isolated DNA molecule comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said polymerase, wherein said mutation is in the 5'-3' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

46. A recombinant host cell comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said polymerase, wherein said mutation is in the 5'-3' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase.

47. A method of producing a mutant *Thermotoga neapolitana* DNA polymerase, said method comprising:


(a) culturing a host cell comprising a gene encoding a mutant *Thermotoga neapolitana* DNA polymerase having a mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said polymerase, wherein said mutation is in the 5'-3' exonuclease domain of said polymerase, and further wherein said mutant *Thermotoga neapolitana* DNA polymerase is a Pol I-type DNA polymerase;

- (b) expressing said gene; and
- (c) isolating said mutant *Thermotoga neapolitana* DNA polymerase from said host cell.

REMARKS

Claims 1-21 were in the Application as filed. Claims 1-21 have been cancelled without prejudice in order to further the Applicants' business interests and the prosecution of the present Application. Applicants reserve the right to prosecute the original claims (or similar claims) in the future. Support for the new claims is found throughout the Application.

Dated: August 18, 2000



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THERMOPHILIC DNA POLYMERASES FROM *THERMOTOGA NEAPOLITANA*

FIELD OF THE INVENTION

The present invention relates to thermostable DNA polymerases derived from the hyperthermophilic eubacteria *Thermotoga neapolitana* and means for isolating and producing the enzymes. Thermostable DNA polymerases are useful in many recombinant DNA techniques, especially thermal cycle sequencing and nucleic acid amplification.

BACKGROUND

Thermophilic bacteria are organisms which are capable of growth at elevated temperatures. Unlike the mesophiles, which grow best at temperatures in the range of 25-40°C, or psychrophiles, which grow best at temperatures in the range of 15-20°C, thermophiles grow best at temperatures greater than 50°C. Indeed, some thermophiles grow best at 65-75°C, and some of the hyperthermophiles grow at temperatures up to 130°C (See e.g., J.G. Black, Microbiology Principles and Applications, 2d edition, Prentice Hall, New Jersey, [1993] p. 145-146).

The thermophilic bacteria encompass a wide variety of genera and species. There are thermophilic representatives included within the phototrophic bacteria (e.g., the purple bacteria, green bacteria, and cyanobacteria), eubacteria (e.g., *Bacillus*, *Clostridium*, *Thiobacillus*, *Desulfotomaculum*, *Thermus*, lactic acid bacteria, actinomycetes, spirochetes, and numerous other genera), and the archaeobacteria (e.g., *Pyrococcus*, *Thermococcus*, *Thermoplasma*, *Thermotoga*, *Sulfolobus*, and the methanogens). There are aerobic, as well as anaerobic thermophilic organisms. Thus, the environments in which thermophiles may be isolated vary greatly, although all of these organisms are always isolated from areas associated with high temperatures. Natural geothermal habitats have a worldwide distribution and are primarily associated with tectonically active zones where major movements of the earth's crust occur.

Thermophilic bacteria have been isolated from all of the various geothermal habitats, including boiling springs with neutral pH ranges, sulfur-rich acidic springs, and deep-sea vents. For all of these organisms, it appears that the organisms present in these geothermal habitats are optimally adapted to the temperatures at which they are living (T.D. Brock, "Introduction: An overview of the thermophiles," in T.D. Brock (ed.), Thermophiles: General, Molecular and Applied Microbiology, John Wiley & Sons, New York [1986], pp. 1-16). Basic as well as applied research on thermophiles has provided some insight into the physiology of these organisms, as well as promise for use of these organisms in industry and biotechnology.

I. The Genus *Thermotoga*

The *Thermotoga* is a recently described genus with three recognized species, which includes the most extremely thermophilic eubacteria known. The genus was first described in 1986, by Huber *et al.*, (R. Huber *et al.* Arch. Microbiol. 144:324 [1986]; and Int. J. Syst. Bacteriol., 36:575 [1986]). At this time, there was only one species described, *T. maritima*. *T. neapolitana* was first described by Jannasch *et al.* in 1986 (Jannasch *et al.*, Arch. Microbiol., 150:103-104 [1986]; and Int. J. Syst. Bacteriol., 39:93 [1989]). *T. thermarum* was described by Windberger *et al.* in 1989 (Windberger *et al.*, Arch. Microbiol., 151:506-512; and Int. J. Syst. Bacteriol., 42:327 [1992]).

These organisms were originally isolated from geothermally heated marine sediments and hot springs. For example, *T. maritima* has been isolated from geothermally heated sea floors in Italy, the Azores, Indonesia, and Iceland as well as from continental, solfataric springs in Africa. *T. neapolitana* has been isolated from a submarine thermal vent near Naples and from continental, solfataric springs in Africa.

Members of the genus *Thermotoga* are considered to be hyperthermophilic, as they are capable of growth at temperatures up to 90°C, although growth will occur at temperatures between 55°C and 90°C; the optimum growth temperature is between 70-80°C. The *Thermotoga* are strictly anaerobic, non-sporing, Gram-negative rods,

that ferment carbohydrates, and may be motile by polar, lateral or peritrichous flagella, although some strains are non-motile. The cells are surrounded by a sheath-like outer structure which usually balloons over the ends. In all species of *Thermotoga*, 1-4 cells may be enclosed within one sheath.

5 *T. maritima*, *T. neapolitana*, and *T. thermarum* are distinct species as judged by the numerous differences. For example, *T. maritima* possesses a single subpolar flagellum and is motile, while motile strains of *T. neapolitana* possess peritrichous flagella (some strains are non-motile), and *T. thermarum* possesses lateral flagella. In addition, *T. neapolitana* will grow in NaCl concentrations ranging from 0.25 to 6.0%,
10 while *T. maritima* will grow in NaCl concentrations ranging from 0.25 to 3.75%, and *T. thermarum* will grow in NaCl concentrations ranging from 0.2-0.55% (J.G. Holt *et al.* (eds.), Bergey's Manual® of Determinative Bacteriology, 9th ed., Williams & Wilkins, Baltimore, [1994], p. 333). Also, there are differences in the susceptibility of these species to rifampicin, and differences in the inhibitory effects of hydrogen and sulfur on these species. Furthermore, the rate of growth at optimum growth
15 temperature (80°C) is a doubling time of about 45 min for *T. neapolitana* and about 75 min for *T. maritima*. The G+C content of the DNA of *T. maritima* and *T. neapolitana* is 46% and 41%, respectively. The DNA from *T. maritima* and *T. neapolitana* shows only about 25-30% homology by DNA-DNA hybridization studies.

20 A few of the enzymes of the *Thermotoga* and other thermophilic genera have been studied in varying degrees of detail. As discussed below, the use of thermophilic enzymes in industry has been viewed as providing advantages over the use of mesophilic enzymes.

25 II. Uses For Thermophilic Enzymes

Advances in molecular biology and industrial processes have led to an increased interest in thermophilic organisms such as *Thermotoga*. Of particular interest has been the development of thermophilic enzymes for use in industrial processes such as the detergent, flavor-enhancing, and starch industries. Indeed, the

cost savings associated with the longer storage stability and higher activity at higher temperatures of thermophilic enzymes, as compared to mesophilic enzymes, provide good reason to select and develop thermophilic enzymes for industrial and biotechnology applications. Thus, there has been much research conducted to characterize enzymes from thermophilic organisms. However, some thermophilic enzymes have less activity than their mesophilic counterparts under similar conditions at the elevated temperatures used in industry (typically temperatures in the range of 50-100°C) (T.K. Ng and William R. Kenealy, "Industrial Applications of Thermostable Enzymes," in T.D. Brock (ed.), Thermophiles: General, Molecular, and Applied Microbiology, [1986], John Wiley & Sons, New York, pp. 197-215). Thus, the choice of a thermostable enzyme over a mesophilic one may not be as beneficial as originally assumed. Nonetheless, of the \$400 million worth of enzymes sold worldwide in 1984, 90% were thermostable enzymes used by the detergent and starch industries (Ng and Kenealy, at p. 206). However, much research remains to be done to characterize and compare thermophilic enzymes of importance in areas such as molecular biology (e.g., polymerases, ligases, topoisomerases, restriction endonucleases, etc.).

III. Thermophilic DNA Polymerases

Extensive research has been conducted on the isolation of DNA polymerases from mesophilic organisms such as *E. coli* (See e.g., Bessman *et al.*, J. Biol. Chem. 223:171 [1957]; Buttin and Kornberg, J. Biol. Chem. 241:5419 [1966]; and Joyce and Steitz, Trends Biochem. Sci., 12:288-292 [1987]). Other mesophilic polymerases have also been studied, such as those of *Bacillus licheniformis* (Stenesh and McGowan, Biochim. Biophys. Acta 475:32-44 [1977]; Stenesh and Roe, Biochim. Biophys. Acta 272:156-166 [1972]); *Bacillus subtilis* (Low *et al.*, J. Biol. Chem., 251:1311 [1976]; and Ott *et al.*, J. Bacteriol., 165:951 [1986]; *Salmonella typhimurium* (Harwood *et al.*, J. Biol. Chem., 245:5614 [1970]; Hamilton and Grossman, Biochem., 13:1885 [1974]), *Streptococcus pneumoniae* (Lopez *et al.*, J. Biol. Chem., 264:4255 [1989]), and

Micrococcus luteus (Engler and Bessman, Cold Spring Harbor Symp., 43:929 [1979]), to name but a few.

Somewhat less investigation has been made on the isolation and purification of DNA polymerases from thermophilic organisms. However, native (*i.e.*, non-recombinant) and/or recombinant thermostable DNA polymerases have been purified from various organisms, as shown in Table 1 below.

TABLE 1

Polymerases Isolation From Thermophilic Organisms

Organism	Citation
<i>Thermus aquaticus</i>	Kaledin <i>et al.</i> , Biochem., 45:494-501 (1980); Biokhimiya 45:644-651 (1980).
	Chien <i>et al.</i> , J. Bacteriol., 127:1550 (1976).
	University of Cincinnati Master's thesis by A. Chien, "Purification and Characterization of DNA Polymerase from <i>Thermus aquaticus</i> ," (1976).
	University of Cincinnati, Master's thesis by D. B. Edgar, "DNA Polymerase From an Extreme Thermophile: <i>Thermus aquaticus</i> ," (1974).
	U.S. Patent No. 4,889,818*
	U.S. Patent No. 5,352,600*
	U.S. Patent No. 5,079,352*
	European Patent Pub. No. 258,017*
	PCT Pub. No. WO 94/26766*
	PCT Pub. No. WO 92/06188*
	PCT Pub. No. WO 89/06691*
<i>Thermotoga maritima</i>	PCT Pub. No. WO 92/03556*

TABLE 1

Polymerases Isolation From Thermophilic Organisms

Organism	Citation
<i>Thermotoga</i> strain FjSS3-B.1	Simpson <i>et al.</i> , Biochem. Cell Biol., 68:1292-1296 (1990).
<i>Thermosipho africanus</i>	PCT Pub. No. 92/06200*
<i>Thermus thermophilus</i>	Myers and Gelfand, Biochem., 30:7661 (1991)
	PCT Pub. No. WO 91/09950*
	PCT Pub. No. WO 91/09944*
	Bechtereva <i>et al.</i> , Nucleic Acids Res., 17:10507 (1989).
	Glukhov <i>et al.</i> , Mol. Cell. Probes 4:435-443 (1990).
<i>Thermus thermophilus</i>	Carballeira <i>et al.</i> , BioTech., 9:276-281 (1990)
	Rüttiman <i>et al.</i> , Eur. J. Biochem., 149:41-46 (1985).
	Oshima <i>et al.</i> , J. Biochem., 75:179-183 (1974).
	Sakaguchi and Yajima, Fed. Proc., 33:1492 (1974) (abstract).
<i>Thermus flavus</i>	Kaledin <i>et al.</i> , Biochem., 46:1247-1254 (1981); Biokhimiya 46:1576-1584 (1981).
	PCT Pub. No. WO 94/26766*
<i>Thermus ruber</i>	Kaledin <i>et al.</i> , Biochem., 47:1515-1521 (1982); Biokhimiya 47:1785-1791 (1982)
<i>Thermoplasma acidophilum</i>	Hamal <i>et al.</i> , Eur. J. Biochem., 190:517-521 (1990).
	Forterre <i>et al.</i> , Can. J. Microbiol., 35:228-233 (1989).

TABLE 1

Polymerases Isolation From Thermophilic Organisms

Organism	Citation
<i>Sulfolobus acidocaldarius</i>	Salhi <i>et al.</i> , J. Mol. Biol., 209:635-641 (1989).
	Salhi <i>et al.</i> , Biochem. Biophys. Res. Comm., 167:1341-1347 (1990).
	Rella <i>et al.</i> , Ital. J. Biochem., 39:83-99 (1990).
	Forterre <i>et al.</i> , Can. J. Microbiol., 35:228-233 (1989).
	Rossi <i>et al.</i> , System. Appl. Microbiol., 7:337-341 (1986).
	Klimczak <i>et al.</i> , Nucleic Acids Res., 13:5269-5282 (1985).
	Elie <i>et al.</i> , Biochim. Biophys. Acta 951:261-267 (1988).
<i>Bacillus caldotenax</i>	J. Biochem., 113:401-410 (1993).
<i>Bacillus stearothermophilus</i>	Sellmann <i>et al.</i> , J. Bacteriol., 174:4350-4355 (1992).
	Stenesh and McGowan, Biochim. Biophys. Acta 475:32-44 (1977).
	Stenesh and Roe, Biochim. Biophys. Acta 272:156-166 (1972).
	Kaboev <i>et al.</i> , J. Bacteriol., 145:21-26 (1981).
<i>Methanobacterium thermoautotrophicum</i>	Klimczak <i>et al.</i> , Biochem., 25:4850-4855 (1986).
<i>Thermococcus litoralis</i>	Kong <i>et al.</i> , J. Biol. Chem. 268:1965 (1993); U.S. Patent No. 5,210,036*; U.S. Patent No. 5,322,785*

TABLE 1

Polymerases Isolation From Thermophilic Organisms

Organism	Citation
<i>Pyrococcus furiosus</i>	Lundberg <i>et al.</i> , Gene 108:1 (1991) PCT Pub. WO 92/09689

* Herein incorporated by reference.

Although the organisms listed in Table 1 are considered thermophiles, many are in the archaebacteria, a group that is evolutionarily distinct from the eubacterial genus *Thermotoga*.

In addition to native forms, modified forms of thermostable DNA polymerases having reduced or absent 5' to 3' exonuclease activity have been expressed and purified from *T. aquaticus*, *T. maritima*, *Thermus species sps17*, *Thermus species Z05*, *T. thermophilus* and *T. africanus* [PCT Publication No. 92/06200].

IV. Uses for Thermophilic DNA Polymerases

One application for thermostable DNA polymerases is the polymerase chain reaction (PCR). The PCR process is described in U.S. Patent Nos. 4,683,195 and 4,683,202, the disclosures of which are incorporated herein by reference. Primers, template, nucleoside triphosphates, the appropriate buffer and reaction conditions, and polymerase are used in the PCR process, which involves denaturation of target DNA, hybridization of primers and synthesis of complementary strands. The extension product of each primer becomes a template for the production of the desired nucleic acid sequence. If the polymerase employed in the PCR is a thermostable enzyme, then polymerase need not be added after each denaturation step because heat will not destroy the polymerase activity. Use of such enzymes as *Taq* DNA polymerase allows repetitive heating/cooling cycles without the requirement of fresh enzyme at each cooling step. This represents a major advantage over the use of mesophilic enzymes such as Klenow, as fresh enzyme must be added to each individual reaction tube at

every cooling step. The use of *Taq* in PCR is disclosed in U.S. Patent No. 4,965,188, EP Publ. No. 258,017, and PCT Publ. No. 89/06691, herein incorporated by reference.

In addition to PCR, *Taq* DNA polymerase is widely used in other molecular biology techniques including recombinant DNA methods. For example, various forms of *Taq* have been used in a combination method which utilizes PCR and reverse transcription (*see e.g.*, U.S. Patent No. 5,322,770, herein incorporated by reference). DNA sequencing methods have also been described which utilize *Taq* (*see e.g.*, U.S. Patent No. 5,075,216, herein incorporated by reference).

However, *Taq* DNA polymerase has certain characteristics which are undesirable in PCR and other applications including the presence of 5' to 3' exonuclease activity. When thermostable DNA polymerases which have 5' to 3' exonuclease activity (*Taq*, *Tma*, *Tsps17*, *TZ05*, *Tth* and *Taf*) are used in the PCR process and other methods, a variety of undesirable results have been observed, including a limitation of the amount of PCR product produced, an impaired ability to generate long PCR products or to amplify regions containing significant secondary structure, the production of shadow bands or the attenuation in signal strength of desired termination bands during DNA sequencing, the degradation of the 5' end of oligonucleotide primers in the context of double-stranded primer-template complex, nick-translation synthesis during oligonucleotide-directed mutagenesis and the degradation of the RNA component of RNA:DNA hybrids. When utilized in a PCR process with double-stranded primer-template complex, the 5' to 3' exonuclease activity of a DNA polymerase may result in the degradation of the 5' end of the oligonucleotide primers. This activity is not only undesirable in PCR, but also in second-strand cDNA synthesis and sequencing processes.

In the choice of enzyme for sequencing, various factors must be considered. For example, large quantities of the enzyme should be easy to prepare; the enzyme

must be stable upon storage for considerable time periods; the enzyme should accept all deoxy and dideoxy nucleotides and analogues as substrates with equal affinities and high fidelity; the polymerase action should be highly processive over nucleotide extensions to 1 kb and beyond, even through regions of secondary structure within the template; the activity should remain high, even in suboptimal conditions; and it should be inexpensive (A.T. Bankier, "Dideoxy sequencing reactions using Klenow fragment DNA polymerase I," in H. and A. Griffin (eds.), Methods in Molecular Biology: DNA Sequencing Protocols, Humana Press, Totowa, NJ, [1993], pp. 83-90). Furthermore the enzyme should be able to function at elevated temperatures (*i.e.*, greater than about 70°C) so that non-specific priming reactions are minimized. However, there are no commercially available enzymes which fully meet all of these criteria. Thus, mutant forms of enzymes have been produced in order to address some of these needs.

For example, mutant forms of thermostable DNA polymerases which exhibit reduced or absent 5' to 3' exonuclease activity have been generated. The Stoffel fragment of *Taq* DNA polymerase lacks 5' to 3' exonuclease activity due to genetic manipulations which result in the production of a truncated protein lacking the N-terminal 289 amino acids (See *e.g.*, Lawyer *et al.*, J. Biol. Chem., 264:6427-6437 [1989]; and Lawyer *et al.*, PCR Meth. Appl., 2:275-287 [1993]). Analogous mutant polymerases have been generated for polymerases derived from *T. maritima*, *Tsps17*, *TZ05*, *Tth* and *Taf*. While the generation of thermostable polymerases lacking 5' to 3' exonuclease activity provides improved enzymes for certain applications, some of these mutant polymerases still have undesirable characteristics including the presence of 3' to 5' exonuclease activity.

The 3' to 5' exonuclease activity is commonly referred to as a proof-reading activity. The 3' to 5' exonuclease removes bases which are mismatched at the 3' end of a primer-template duplex. While the presence of 3' to 5' exonuclease activity may

be advantageous as it leads to an increase in the fidelity of replication of nucleic acid strands it also has other undesirable characteristics. The 3' to 5' exonuclease activity found in thermostable DNA polymerases such as *Tma* (including mutant forms of *Tma* that lack 5' to 3' exonuclease activity) also degrades single-stranded DNA such as the primers used in the PCR, single-stranded templates and single-stranded PCR products. The integrity of the 3' end of an oligonucleotide primer used in a primer extension process (e.g., PCR, Sanger sequencing methods, etc.) is critical as it is from this terminus that extension of the nascent strand begins. Degradation of the 3' end leads to a shortened oligonucleotide which in turn results in a loss of specificity in the priming reaction (i.e., the shorter the primer the more likely it becomes that spurious or non-specific priming will occur).

The degradation of an oligonucleotide primer by a 3' exonuclease can be prevented by the use of modified nucleotides at the 3' terminus. For example, the use of dideoxynucleotides or deoxynucleotides having a phosphorothiolate linkage at the 3' terminus of an oligonucleotide would prevent degradation by 3' exonucleases. However, the need to use modified nucleotides to prevent degradation of oligonucleotides by 3' exonuclease increases the time and cost required to prepare oligonucleotide primers.

A few examples of a thermostable polymerase which lack both 5' to 3' exonuclease and 3' to 5' exonuclease are known. As discussed above, the Stoffel fragment of *Taq* DNA polymerase lacks the 5' to 3' exonuclease activity due to genetic manipulation and no 3' to 5' activity is present as *Taq* polymerase is naturally lacking in 3' to 5' exonuclease activity. Likewise the *Tth* polymerase naturally lacks 3' to 5' exonuclease activity and genetic deletion of N-terminal amino acids removes the 5' to 3' exonuclease activity.

Despite the development of recombinant enzymes such as Stoffel fragment, there remains a need for other thermostable polymerases having improved characteristics. For example, thermostable polymerases are used in Sanger dideoxynucleotide sequencing protocols. The most commonly used enzyme is *Taq* polymerase or a modified form of *Taq* polymerase. High concentrations of the expensive dideoxynucleotides must be used in the sequencing reaction when these enzymes are employed as they have a fairly low affinity for dideoxynucleotides. The art needs a thermostable polymerase which displays a higher affinity for dideoxynucleotides as this would result in considerable cost savings. In addition, the art needs additional thermostable polymerases having novel properties to improve the results obtained when using techniques such as DNA amplification, sequencing and nick-translation.

SUMMARY OF THE INVENTION

The present invention relates to purified thermostable DNA polymerases derived from the eubacteria *Thermotoga neapolitana* (*Tne*). Nucleic acid sequences encoding the full-length *Tne* DNA polymerase is provided. In addition, nucleic acid sequences encoding several modified forms of the *Tne* DNA polymerase are provided herein. The present invention provides methods for the isolation of purified preparations of *Tne* DNA polymerases. The *Tne* DNA polymerases may be isolated from *Thermotoga neapolitana* cells or from host cells containing nucleic sequences encoding a *Tne* DNA polymerase.

In one embodiment, the present invention contemplates a purified thermostable DNA polymerase derived from the eubacterium *Thermotoga neapolitana* which is capable of DNA synthetic activity. In another embodiment, the purified *Tne* DNA polymerase has 3' exonuclease activity. In yet another embodiment, the purified *Tne*

DNA polymerase has 5' exonuclease activity. In one preferred embodiment, the purified *Tne* DNA polymerase comprises the amino acid sequence of SEQ ID NO:2. In a particularly preferred embodiment, the specific activity of the synthetic activity of the purified *Tne* DNA polymerase is approximately 100,000 units/mg.

5 In another embodiment, the purified thermostable *Tne* DNA polymerase is a non-naturally occurring or recombinant DNA polymerase. The recombinant *Tne* DNA polymerase may further contain 3' exonuclease activity and/or 5' exonuclease activity.

In a preferred embodiment, the non-naturally occurring *Tne* DNA polymerase has reduced levels of 3' exonuclease activity. In another embodiment, the
10 non-naturally occurring *Tne* DNA polymerase lacks significant 5' exonuclease activity. In a particularly preferred embodiment, the non-naturally occurring *Tne* DNA polymerase comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 8, 16, 19, 23, 26, 29, 33 and 35.

The present invention provides nucleic acid sequences encoding thermostable
15 DNA polymerases. In a preferred embodiment, an oligonucleotide comprising the nucleic acid sequence of SEQ ID NO:1 encodes the thermostable DNA polymerase. These nucleic acid sequences encoding thermostable DNA polymerases may be modified to encode a polymerase which lacks significant 5' exonuclease activity. In a preferred embodiment, the modified nucleic acid sequences encoding a thermostable
20 DNA polymerase comprise the nucleotide sequence of SEQ ID NO:7. In one embodiment, the polymerase encoded by the modified nucleic acid sequences displays reduced levels of 3' exonuclease activity. In a particularly preferred embodiment, the modified nucleic acid sequences encoding a polymerase having reduced levels of 3' exonuclease activity are selected from the group consisting of SEQ ID NOS:7, 15, 18, 22, 25, 28, 32 and 34.
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5 The present invention provides recombinant DNA vectors containing nucleic acid sequences which encode a thermostable DNA polymerase having DNA synthetic activity. In a preferred embodiment the polymerase-encoding nucleic acid sequences are set forth in SEQ ID NO:1. The recombinant DNA vector may contain a modified nucleic sequence encoding a thermostable DNA polymerase which lacks significant 5' exonuclease activity. In a preferred embodiment, the recombinant DNA vector contains a modified nucleic acid sequence which comprises SEQ ID NO:7.

10 In a preferred embodiment, the recombinant DNA vector contains modified nucleic acid sequences encoding a thermostable DNA polymerase which exhibits reduced levels 3' exonuclease activity. In a particularly preferred embodiment, the modified sequences encoding a thermostable DNA polymerase which exhibits reduced levels 3' exonuclease activity are selected from the group consisting of SEQ ID NOS:7, 15, 18, 22, 25, 28, 32 and 34.

15 The present invention further contemplates the transformation of host cells with the recombinant DNA vectors containing nucleic acid sequences encoding *The* DNA polymerases. The invention is not limited by the choice of host cell; host cells may comprise procaryotic or eucaryotic cells. In a preferred embodiment, the host cell is an *E. coli* host cell.

20 The invention further provides methods for determining the DNA sequence of a segment or portion of a DNA molecule using the *The* DNA polymerases of the invention. Dideoxynucleotide (ddNTP) chain termination sequencing protocols are used in conjunction with the polymerases of the invention. Traditional (*i.e.*, Sanger) as well as other methods, including but not limited to, chain termination sequencing or thermal cycle sequencing protocols benefit from the use of the *The* DNA polymerases of the invention. The claimed *The* DNA polymerases have a high affinity for dideoxynucleotides; accordingly the following ratios of dNTPs and ddNTPs are

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contemplated for use in either thermal cycling or Sanger chain termination protocols when *Tne* DNA polymerases are employed: dATP:ddATP is 1:1.67 \pm 50%; dCTP:ddCTP is 1:0.83 \pm 50%; dGTP:ddGTP is 1:0.67 \pm 50% and TTP:ddTTP is 1:2.5 \pm 50% where each dNTP is present at a final concentration of about 1 μ M to 120 μ M.

DESCRIPTION OF THE DRAWINGS

Figure 1 provides a schematic representation of the 5' exonuclease, 3' exonuclease and polymerase domains in several DNA polymerases.

Figure 2 shows an alignment of amino acid residues from three regions within the 3' exonuclease domain of selected DNA polymerases.

Figure 3 shows the alignment of the amino acid residues (using the one letter code for the amino acids) from *E. coli* DNA polymerase I, *Tma* DNA polymerase and *Tne* DNA polymerase.

Figure 4 provides a schematic depicting the full length and mutant *Tne* DNA polymerases of the present invention.

Figure 5A shows an autoradiograph of a sequencing gel.

Figure 5B shows an autoradiograph of a sequencing gel.

Figure 6A shows an autoradiograph of a sequencing gel.

Figure 6B shows an autoradiograph of a sequencing gel.

Figure 6C shows an autoradiograph of a sequencing gel.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product. The wild-type form of the coding region for the *Tne* DNA polymerase is listed in SEQ ID NO:1; the wild-type form of the *Tne* DNA polymerase protein is listed in SEQ ID NO:2. The *Tne* DNA polymerase proteins encoded by "modified" or "mutant" genes are referred to as non-naturally occurring *Tne* DNA polymerases.

The term "recombinant DNA vector" as used herein refers to DNA sequences containing a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism. DNA sequences necessary for expression in procaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals and enhancers.

As used herein, the terms "cell," "cell line," and cell culture" are used interchangeably and all such designations include progeny. The words "transformants" or "transformed cells" include the primary transformed cell and cultures derived from that cell without regard to the number of transfers. All progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included in the definition of transformants.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector."

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in procaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eucaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "transfection" as used herein refers to the introduction of foreign DNA into eucaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation,

DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementary may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementary between the nucleic acids. The degree of complementary between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementary. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second

target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Low stringency conditions comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

The art knows well that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions which promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.).

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

5 As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

10 As used herein, the term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m .

15 20 As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, 25 conditions of "weak" or "low" stringency are often required with nucleic acids that are

derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195 and 4,683,202, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target

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molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target

sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q β replicase, MDV-1 RNA is the specific template for the replicase [D.L. Kacian *et al.*, *Proc. Natl. Acad. Sci USA* 69:3038 (1972)]. Other nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters [M. Chamberlin *et al.*, *Nature* 228:227 (1970)]. In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides where there is a mismatch between the oligonucleotide substrate and the template at the ligation junction [D.Y. Wu and R. B. Wallace, *Genomics* 4:560 (1989)]. Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences [*PCR Technology*, H.A. Erlich (ed.) (Stockton Press 1989)].

As used herein, the terms "PCR product", "PCR fragment" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for

primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, the term "an oligonucleotide having a nucleotide sequence encoding a gene" means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, *etc.* (defined *infra*).

Transcriptional control signals in eucaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription [Maniatis, T. *et al.*, *Science* 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of eucaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in procaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eucaryotic promoters and enhancers have a broad host range while others are functional in a

limited subset of cell types [for review see Voss, S.D. *et al.*, *Trends Biochem. Sci.*, 11:287 (1986) and Maniatis, T. *et al.*, *supra* (1987)]. For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells [Dijkema, R. *et al.*, *EMBO J.* 4:761 (1985)]. Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1 α gene [Uetsuki, T. *et al.*, *J. Biol. Chem.*, 264:5791 (1989), Kim, D.W. *et al.*, *Gene* 91:217 (1990) and Mizushima, S. and Nagata, S., *Nuc. Acids. Res.*, 18:5322 (1990)] and the long terminal repeats of the Rous sarcoma virus [Gorman, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 79:6777 (1982)] and the human cytomegalovirus [Boshart, M. *et al.*, *Cell* 41:521 (1985)].

As used herein, the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (*i.e.*, the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript in eucaryotic host cells. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site [Sambrook, J. *et al.*, *Molecular Cloning: A*

Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp. 16.7-16.8]. A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eucaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript.

Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam*HI/*Bcl*II restriction fragment and directs both termination and polyadenylation [J. Sambrook, *supra*, at 16.6-16.7].

Eucaryotic expression vectors may also contain "viral replicons" or "viral origins of replication." Viral replicons are viral DNA sequences which allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors which contain either the SV40 or polyoma virus origin of replication replicate to high copy number (up to 10^4 copies/cell) in cells that express the appropriate viral T antigen. Vectors which contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number (~100 copies/cell).

The *Tne* polymerases may be expressed in either procaryotic or eucaryotic host cells. Nucleic acid encoding the *Tne* polymerase may be introduced into bacterial host cells by a number of means including transformation of bacterial cells made competent for transformation by treatment with calcium chloride or by electroporation. If the *Tne* polymerases are to be expressed in eucaryotic host cells, nucleic acid encoding the *Tne* polymerase may be introduced into eucaryotic host cells by a number of means including calcium phosphate co-precipitation, spheroplast fusion, electroporation and the like. When the eucaryotic host cell is a yeast cell, transformation may be affected by treatment of the host cells with lithium acetate or by electroporation.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNA s which encode a multitude of proteins. However, isolated nucleic acid encoding a *Tne* polymerase includes, by way of example, such nucleic acid in cells ordinarily expressing a *Tne* polymerase where the nucleic acid is in a

chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid or oligonucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid or oligonucleotide is to be utilized to express a protein, the oligonucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide may single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide may be double-stranded).

As used herein the term "coding region" when used in reference to structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, recombinant *The* DNA polymerases are expressed in bacterial host cells and the polymerases are purified by the removal of host cell proteins; the percent of recombinant *The* DNA polymerase is thereby increased in the sample.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" is used herein to indicate a protein isolated from a naturally occurring (*i.e.*, a nonrecombinant) source. Molecular biological techniques may be used to produce a recombinant form of a protein which has identical properties

when compared to the native form of the protein. The term "*rTne*" is used to designate a recombinant form of *Tne* polymerase. The terms "*nTne*" and "*nTaq*" are used to designate the native forms of *Tne* polymerase and *Taq* polymerase, respectively.

5 As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

10 As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, *Tne* DNA polymerases and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-*Tne* polymerase protein). The fusion partner may enhance solubility of the *Tne* polymerase protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If
15 desired, the fusion protein may be removed from the protein of interest (*i.e.*, *Tne* DNA polymerase or fragments thereof) by a variety of enzymatic or chemical means known to the art.

20 The term "5' exonuclease activity" refers to the presence of an activity in a protein which is capable of removing nucleotides from the 5' end of an oligonucleotide. 5' exonuclease activity may be measured using any of the assays provided herein.

25 The term "3' exonuclease activity" refers to the presence of an activity in a protein which is capable of removing nucleotides from the 3' end of an oligonucleotide. 3' exonuclease activity may be measured using any of the assays provided herein.

The terms "DNA polymerase activity," "synthetic activity" and "polymerase activity" are used interchangeably and refer to the ability of an enzyme to synthesize new DNA strands by the incorporation of deoxynucleoside triphosphates. The examples below provide assays for the measurement of DNA polymerase activity.

5 The term "reduced levels of 3' exonuclease" is used in reference to the level of 3' exonuclease activity displayed by the wild-type *Tne* DNA polymerase (*i.e.*, the polymerase of SEQ ID NO:2) and indicates that the modified or "non-naturally occurring" polymerase exhibits lower levels of 3' exonuclease than does the full-length or unmodified enzyme.

10 The phrase "lacks significant 5' exonuclease activity" is used relative to the level of 5' exonuclease activity displayed by the wild-type *Tne* DNA polymerase (*i.e.*, the polymerase of SEQ ID NO:2) and indicates that the modified or "non-naturally occurring" polymerase exhibits such low levels of 5' exonuclease that the measurement is at background levels in the assay.

15 DESCRIPTION OF THE INVENTION

The present invention provides purified thermostable DNA polymerase I enzymes derived from *Thermotoga neapolitana* (*Tne*). These thermostable enzymes comprise the wild-type form of the enzyme as well as mutant forms which possess altered characteristics relative to the wild-type enzyme. In particular, the present invention provides deletion mutants which lack 5' exonuclease activity. Further the present invention provides modified forms of *Tne* DNA polymerases which lack 5' exonuclease activity and have reduced or absent 3' exonuclease activity.

20 The present invention also relates to an improved method of determining the nucleic sequence of a DNA molecule using chain terminating dideoxynucleotides in conjunction with the modified *Tne* DNA polymerases. The novel properties of the

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polymerases of the invention provide improved enzymes for a variety of applications which utilize thermostable DNA polymerases.

The description of the invention is divided into: I. General Structural Features of Type A DNA Polymerases, II. Generation of *Tne* DNA Polymerases, III. Use of *Tne* DNA Polymerases in the PCR and IV. Use of *Tne* DNA Polymerases in DNA Sequencing Methods.

I. General Structural Features Of DNA Polymerases

DNA polymerases (DNAPs), such as those isolated from *E. coli* or from thermophilic bacteria of the genera *Thermus* or *Thermotoga*, are enzymes that synthesize new DNA strands. Several of the known DNAPs contain associated nuclease activities in addition to the synthetic or polymerization activity of the enzyme.

Some DNAPs are known to remove nucleotides from the 5' and 3' ends of DNA chains [Kornberg, *DNA Replication*, W.H. Freeman and Co., San Francisco, pp. 127-139 (1980)]. These nuclease activities are usually referred to as 5' exonuclease and 3' exonuclease activities, respectively. For example, the 5' exonuclease activity located in the N-terminal domain of several DNAPs participates in the removal of RNA primers during lagging strand synthesis during DNA replication and the removal of damaged nucleotides during repair. Some DNAPs, such as the *E. coli* DNA polymerase, also have a 3' exonuclease activity responsible for proof-reading during DNA synthesis (Kornberg, *supra*).

DNAPs isolated from *Thermus aquaticus* (*Taq*), *Thermus flavus* (*Tfl*) and *Thermus thermophilus* (*Tth*) have a 5' exonuclease activity, but lack a functional 3' exonucleolytic domain [Tindall and Kunkell, *Biochem.* 27:6008 (1988)]. However, the lack of a 3' exonuclease domain is not a general feature of DNAPs derived from

thermophilic bacteria as DNA polymerases from the thermophiles *Thermotoga maritima* (*Tma*), *Bacillus caldotenax*, *Thermococcus litoralis* (*Tli*) and *Pyrococcus furiosus* (*Pfu*) do contain 3' exonuclease activity.

The 5' nuclease activity associated with a number of eubacterial Type A DNA polymerases has been found to reside in the one-third N-terminal region of the protein as an independent functional domain. In these polymerase molecules, the C-terminal two-thirds of the molecule constitute the polymerization domain which is responsible for the synthesis of DNA. Some Type A DNA polymerases also have a 3' exonuclease activity associated with the two-third C-terminal region of the molecule. Figure 1 provides a schematic showing the location of the 5' exonuclease, 3' exonuclease and polymerase domains of a number of eubacterial DNAPs. As noted above, not all DNAPs contain both 5' and 3' exonuclease domains.

Figure 1 provides a schematic depicting the arrangement of the 5' exonuclease ("5' EXO"), 3' exonuclease ("3' EXO") and polymerase ("POL") domains in the DNA polymerases from phage T4 ("φT4"), phage T7 ("φT7"), *E. coli* (DNA polymerase I; "Eco Pol I"), *T. aquaticus* ("Taq"), *T. maritima* ("Tma") and *T. neapolitana* ("Tne"). The absence of a 3' exonuclease domain in *Taq* DNA polymerase is indicated by the use of the line between the boxed 5' exonuclease and polymerase domains; the absence of a 5' nuclease domain in phage T4 polymerase is indicated by the absence of the term "5' EXO" in the first boxed region of the molecule.

The 5' exonuclease activity and the polymerization activity of DNAPs have been separated by proteolytic cleavage or genetic manipulation of the polymerase molecule. The Klenow or large proteolytic cleavage fragment of *E. coli* DNA polymerase I contains the polymerase and 3' exonuclease activity but lacks the 5' nuclease activity [Brutlag *et al.*, *Biochem. Biophys. Res. Commun.* 37:982 (1969)]. The Stoffel fragment of DNAP *Taq* lacks the 5' nuclease activity due to a genetic

manipulation which deleted the N-terminal 289 amino acids of the polymerase molecule [Erlich *et al.*, *Science* 252:1643 (1991)].

The removal of the 5' exonuclease domain from a DNAP may effect the activity of the remaining domains. For example, removal of the 5' exonuclease domain from the *E. coli* polymerase I protein to generate the Klenow fragment affects the fidelity of the remaining large polymerase domain. The fidelity of a DNA polymerase involves several functions including the ability to discriminate against errors when nucleotides are initially inserted, discriminate against extension from misaligned or mispaired primer termini and exonucleolytic removal of errors.

In comparison to the full-length enzyme, the Klenow fragment exhibits altered base substitution error specificity and is less accurate for minus one base frameshift errors at reiterated template nucleotides [Bebenek *et al.*, *J. Biol. Chem.* 265:13878 (1990)]. Thus, the removal of the 5' exonuclease domain of *E. coli* DNA polymerase I adversely affects the fidelity of the remaining 3' exonuclease and synthetic domains.

Removal of a 5' exonuclease domain does not always adversely affect the fidelity of the resultant polymerase fragment. KlenTaq, a truncated version of *Taq* DNA polymerase lacks the first 235 N-terminal amino acids (which includes the 5' exonuclease domain) has been reported improved the fidelity of the polymerase two-fold [Barnes, *Gene* 112:29 (1992)].

Comparison of amino acid sequence in the 3' exonuclease domain of a number DNAPs has identified three domains, termed Exo I-III, which are highly conserved between a variety of mesophilic and thermophilic organisms [Bernad *et al.* *Cell* 59:219 (1989)]. Figure 2 provides a schematic drawing which aligns the amino acid residues from a number of DNAPs over the 3' exonuclease domain. In Figure 2, the one letter code is used for the amino acids; the numbers represent the amino acid residue in a given polymerase. In Figure 2, residues which are highly conserved are indicated by

the use of white letters within a black box. Portions of the 3' exonuclease domain of following polymerases are shown: *Bacillus subtilis* (*Bsu*) polymerase III; *E. coli* (*Eco*) polymerase III ϵ ; phage T4, phage T7, *E. coli* polymerase I, *T. maritima* (*Tma*) polymerase and *T. neapolitana* (*Tne*) polymerase. The "V" indicates amino acid residues involved in single strand DNA binding; the "Δ" indicates amino acid residues involved in metal binding and catalysis.

Site-directed mutagenesis experiments have identified a subset of these conserved residues as being critical for 3' exonuclease activity in *E. coli* polymerase I. The critical residues include D355, D424, D501 which are known to bind divalent metal ions and are essential for 3' exonuclease activity; mutation of these residues reduces 3' exonuclease activity several thousand fold. L361, F473 and Y497 are also important for 3' exonuclease activity and are believed to ensure correct positioning of the substrate in the active site. Mutation of L361 and Y497 reduces 3' exonuclease activity 12.5 to 25-fold; mutation of F473 reduces 3' exonuclease activity about 3000-fold.

PCT Publ. No. WO 92/03556 states that three characteristic domains are critical for 3' exonuclease activity in thermostable DNA polymerases; however, no site-directed mutagenesis is shown for any of the "critical" residues and no 3' exonuclease activity is reported for any of the mutant forms of *Tma* DNA polymerase (primarily deletion mutants) shown. The three domains identified in PCT Publ. No. WO 92/03556 are Domain A, which comprises D-X-E-X³-L; Domain B, which comprises N-X³-D-X³-L and Domain C, which comprises Y-X³-D where X^N represents the number (N) of non-critical amino acids between the specified amino acids. As shown in Figure 2, the location, sequence and spacing of these three domains found in polymerases derived from thermophilic organisms is consistent with the three domains identified in polymerases derived from mesophilic organisms.

While identification of residues which are highly conserved between a number of species provides a starting point for the design of site-directed mutagenesis experiments, it does not provide an absolute prediction of the effect of a given mutation in a particular protein. For example, the present invention shows that substitution of the aspartate at position 468 of the *Tne* DNA polymerase with an asparagine virtually eliminates the 3' exonuclease activity [*Tne* M284(D468N)]. The analogous mutation in the Klenow fragment of DNA polymerase I (D501N) reduces 3' exonuclease activity only by 2-fold [Derbyshire *et al.*, EMBO J. 10:17 (1991)]. These results underscore the fact that much remains to be learned about structure-function relationships and that one cannot predict, with certainty, the effect of a given mutation based on analogy to other proteins.

II. Generation Of *Tne* DNA Polymerases

The present invention provides wild-type and modified forms of *Tne* DNA polymerases. The modified forms lack 5' exonuclease activity and some modified forms also display reduced or absent 3' exonuclease activity.

By the term "reduced or absent 3' exonuclease activity" it is meant that the modified enzyme has less than the level of 3' exonuclease activity found in the wild-type or unmodified enzyme whose protein sequence is listed in SEQ ID NO:2. The modified *Tne* polymerases of the present invention are advantageous in situations where the polymerization (*i.e.*, synthetic) activity of the enzyme is desired but the presence of 5' exonuclease and/or 3' exonuclease activity is not.

The present invention is not intended to be limited by the nature of the alteration (*e.g.*, deletion, insertion, substitution) necessary to render the *Tne* polymerase deficient in 5' exonuclease or 3' exonuclease activity. The present invention

contemplates a variety of methods, including but not limited to proteolysis and genetic manipulation.

1. Reduction Of Exonuclease Activity By Proteolysis

The DNA polymerases having a reduced level of either or both 5' exonuclease and 3' exonuclease activity are produced according to the present invention by physically cleaving the unmodified enzyme with proteolytic enzymes to produce fragments of the enzyme that are deficient in 5' and/or 3' exonuclease activity but retain synthetic activity. The proteolysis can remove the N-terminal one third of the protein (about residues 1 to 297 in SEQ ID NO:2) to remove 5' exonuclease activity. Proteolytic cleavage which removes all or a portion of the 3' exonuclease domain (about residues 298 to 482 in SEQ ID NO:2) will render the resulting enzyme deficient in 3' exonuclease activity. Following proteolytic digestion, the resulting fragments are separated by standard chromatographic techniques and assayed for the ability to synthesize DNA and to act as a 5' or 3' exonuclease. The assays to determine synthetic activity and 5' and 3' exonuclease activity are described in the experimental sections below.

2. Reduction Of Exonuclease Activity By Genetic Manipulation

The examples below describe preferred methods for creating a construct (*i.e.*, a vector) encoding a polymerase derived from *The* DNA polymerase I. The wild-type *The* polymerase is cloned by isolating genomic DNA using molecular biological methods from *T. neapolitana* cells. The genomic DNA is cleaved into fragments about 3 kb or larger using restriction enzymes and the fragments are inserted into a suitable cloning vector such as a plasmid or bacteriophage vector; the vectors

containing fragments of *T. neapolitana* genomic DNA are then transformed into a suitable *E. coli* host. Clones containing DNA encoding the *Tne* polymerase may be isolated using functional assays (*i.e.*, presence of thermostable polymerase in lysates of transformed cells) or by hybridization using a probe derived from a region of conservation among DNA polymerases derived from thermostable organisms. Alternatively, the *T. neapolitana* genomic DNA may be used as the target in a polymerase chain reaction (PCR) where the primers are selected from regions of high sequence conservation among the genes encoding thermostable DNA polymerases. Such a PCR may not amplify the entire coding region of the *Tne* polymerase I gene; in such a case, the full-length *Tne* gene could be isolated by using the amplified fragment as a probe to screen a genomic library containing *T. neapolitana* DNA.

Once the full-length *Tne* polymerase gene is obtained, regions encoding the 5' exonuclease and/or 3' exonuclease may be altered by a variety of means to reduce or eliminate these activities. Suitable deletion and site-directed mutagenesis procedures are described below in the examples.

Deletion of amino acids from the protein can be done either by deletion of the encoding genetic material, or by introduction of a translational stop codon by mutation or frame shift. In addition, proteolytic treatment of the protein molecule can be performed to remove segments of the protein.

In the examples below, specific alterations of the *Tne* polymerase gene were: a deletion between residues 1-849, a deletion between residues 1-945, a deletion between residues 1-966, deletion between residues 1-966, a deletion between residues 1-849 and residues 925-1272 and substitutions at residues 946, 947, 967, 968, 969, 975, 1166, 1167, 1391, 1402, 1407 and 1410. These modified sequences are described below in the examples and at SEQ ID NOS:7, 10, 15, 18, 22, 25, 28, 32, 34, 36 and 38.

Those skilled in the art know that single base changes can be innocuous in terms of enzyme structure and function. Similarly small additions and deletions can be present without substantially changing the exonuclease or polymerase function of the wild-type or modified *Tne* DNA polymerases. To test whether a particular change is innocuous in terms of the effect upon enzymatic activity, the polymerase encoded by a given DNA sequence is tested for the presence of synthetic activity, 5' exonuclease activity and 3' exonuclease activity as in the assays described in the examples below. DNA sequence which contain alterations other than those listed in SEQ ID NOS:7, 10, 15, 18, 22, 25, 28, 32, 34, 36 and 38 but which encode a polymerase molecule having the properties associated with the polymerases encoded by the above SEQ ID NOS are contained within the present invention.

Other deletions and substitutions are also suitable to create modified *Tne* DNA polymerases lacking 5' and/or 3' exonuclease activity. For example, given the degeneracy of the genetic code, several DNA sequences may be used to introduce substitutions which result in the expression of the same amino acid. It is preferable that the alteration decrease the 5' and/or 3' exonuclease activity to a level which is low enough to provide an improved enzyme for a variety of applications such as PCR and chain termination sequencing (including thermal cycle sequencing) as discussed below in the examples. These modifications will preferably not reduce the synthetic activity of the modified enzyme. Modified polymerases are tested for the presence of synthetic activity and 5' and 3' exonuclease activity as in assays described below. Thoughtful consideration of these assays allows for the screening of candidate enzymes whose structure is heretofore as yet unknown. In other words, construct "X" can be evaluated according to the protocol described below to determine whether it is a member of the genus of modified *Tne* polymerases of the present invention as defined functionally, rather than structurally.

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5 The present invention contemplates that the nucleic acid construct of the present invention be capable of expression in a suitable host. In particular it is preferable that the expression system chosen utilize a tightly controlled promoter such that expression of the *Tne* polymerase is prevented until expression is induced. In this manner, potential problems of toxicity of the expressed polymerases to the host cells (and particularly to bacterial host cells) is avoided. Those in the art know methods for attaching various promoters and 3' sequences to a gene structure to achieve efficient and tightly controlled expression. The examples below disclose a number of suitable vectors and vector constructs. Of course, there are other promoter/vector combinations that would be suitable. The choice of a particular vector is also a function of the type of host cell to be employed (*i.e.*, procaryotic or eucaryotic).

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15 It is not necessary that a host organism be used for the expression of the nucleic acid constructs of the invention. For example, expression of the protein encoded by a nucleic acid construct may be achieved through the use of a cell-free in vitro transcription/translation system. An example of such a cell-free system is the commercially available TnT™ Coupled Reticulocyte Lysate System (Promega; this cell-free system is described in U.S. Patent No. 5,324,637, the disclosure of which is herein incorporated by reference).

20 The nucleic acid construct containing DNA encoding the wild-type or a modified *Tne* polymerase may provide for the addition of exogenous sequences (*i.e.*, sequences not encoded by the *Tne* polymerase coding region) to either the 5' or 3' end of the *Tne* polymerase coding region to allow for ease in purification of the resulting polymerase protein (the resulting protein containing such an affinity tag is termed a fusion protein). Several commercially available expression vectors are available which provide for the addition of affinity tags (an example of an exogenous sequence) to either the amino or carboxy-termini of a coding region; in general these affinity tags

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are short stretches of amino acids which do not alter the characteristics of the protein to be expressed (*i.e.*, no change to enzymatic activities).

For example, the pET expression system (Novagen) utilizes a vector containing the T7 promoter which encodes the fusion protein containing a short stretch of histidine residues at either end of the protein and a host cell which can be induced to express the T7 DNA polymerase (*i.e.*, a DE3 host strain). The production of fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein). Additional expression systems which utilize other affinity tags are known to the art.

Once a suitable nucleic acid construct has been made, the *Tne* polymerase may be produced from the construct. The examples below and standard molecular biological teachings enable one to manipulate the construct by different suitable methods.

Once the desired *Tne* polymerase has been expressed, the polymerase is tested for both synthetic and exonuclease activity as described below.

III. Use Of *Tne* DNA Polymerases In The PCR

The wild-type and modified *Tne* polymerases of the present invention provide suitable and in some cases superior enzymes for use in the PCR. As shown in the examples below, the wild-type and modified forms of *Tne* polymerase were found to

require the use of fewer units of polymerase activity to produce a given amount of product DNA in PCRs as compared to wild-type *Taq* DNA polymerase (*i.e.*, *nTaq*) or a modified form of *Tma* DNA polymerase (*i.e.*, *Ultma*TM). In addition, modified forms of *The* polymerase were found to tolerate a broader range of dNTP concentrations and a broader range of magnesium ion concentrations in the PCR. The ability to tolerate a broad range of dNTP is important as it allows flexibility in the range of dNTPs to be used in a reaction; additionally, the ability to tolerate a wide range of dNTP concentrations demonstrates that the enzymes of the invention provide for a robust PCR (*i.e.*, the enzyme is not sensitive to small variations in dNTP concentration). The ability to produce only specific amplification products over a wide range of magnesium ion concentration is advantageous for use in multiplexing PCR reactions.

Several of the modified *The* polymerases provide enzymes having greater resistance to thermal inactivation as compared to *nTaq* or *Ultma*TM DNA polymerases. Greater thermal stability is important for PCR applications as the greater the thermal stability of the enzyme, the fewer units of enzyme must be used in the PCR.

IV. Use Of *The* DNA Polymerases In DNA Sequencing Methods

The sequence of a deoxyribonucleic acid molecule can be elucidated using chemical [Maxam and Gilbert, *Proc. Natl. Acad. Sci. USA* 74:560 (1977)] or enzymatic [Sanger *et al.*, *Proc. Natl. Acad. Sci. USA* 74:5463 (1977)] methods. The enzymatic method of sequencing is based on the ability of a DNA polymerase to extend a primer, hybridized to the template that is to be sequenced, until a chain-terminating nucleotide is incorporated (referred to as chain terminating sequencing). Each sequence determination is carried out as a set of four separate reactions, each of which contains all four deoxyribonucleoside triphosphates (dNTP)

supplemented with a limiting amount of a different dideoxyribonucleoside triphosphate (ddNTP). Because ddNTPs lack the 3'-OH group necessary for chain elongation, the growing oligonucleotide is terminated selectively at G, A, T, or C, depending on the respective dideoxy analog in the reaction.

5 The relative concentrations of each of the dNTPs and ddNTPs can be adjusted to give a nested set of terminated chains over several hundred to a few thousand bases in length. The resulting fragments, each with a common origin but ending in a different nucleotide, are separated according to size by high-resolution denaturing gel electrophoresis.

10 Incorporation of a radiolabel into the oligonucleotide chain permits the visualization of the sequencing products by autoradiography. The end-labeled primer protocol, a modification of that described by Heiner *et al.* [(1988) Applied Biosystems, Inc. DNA Sequencer Model 370 User Bulletin-Taq Polymerase: Increased Enzyme Versatility in DNA Sequencing], uses [γ -³²P]ATP, [γ -³³P]ATP or [γ -³⁵S]ATP to label the sequencing primer. The DNA template and labeled primer are repeatedly annealed and enzymatically extended/terminated in thermal cycled sequencing. The end-labeled primer protocol is the most versatile sequencing method and is useful when working with lambda DNA [Kaledin *et al.*, *Biokhimiya* 45:494 (1980)], PCR templates, and any template where false priming may be a problem. This protocol generates sequence data very close to the primer and is recommended when this is needed. The reaction also contains deaza nucleotide mixes that substitute 7-deaza dGTP for dGTP. The deaza mixes resolve band compressions associated with GC-rich regions [Mizusawa *et al.*, *Nucl. Acids Res.* 14:1319 (1986) and Barr *et al.*, *Biotechniques* 4:428 (1986)].

20 Thermal cycled sequencing is an alternative method for enzymatic sequence analysis which takes advantage of the intrinsic properties of thermophilic DNA polymerases, such as the one isolated from *Thermus aquaticus* (Taq DNA polymerase).

Because the protocol utilizes a thermocycling apparatus, several advantages are realized over conventional sequencing strategies. First, the protocol yields a linear amplification of the template DNA, reducing the amount of template required to achieve a detectable sequence ladder. Using a ^{32}P end-labeled primer, greater than 500 bases of sequence can be obtained from as little as 4 fmol (4×10^{-15} moles) of template after an overnight exposure. Secondly, the high temperatures employed during each denaturation cycle eliminate the requirement for alkaline denaturation and ethanol precipitation of double-stranded DNA (dsDNA) templates. The denaturation cycles also help to circumvent the problems associated with rapid reannealing of linear dsDNA templates such as PCR reaction products. Third, high annealing temperatures increase the stringency of primer hybridization. Fourth, the high polymerization temperature decreases the secondary structure of DNA templates and thus permits polymerization through highly structured regions [Innis *et al.*, Proc. Natl. Acad. Sci USA 85:9436 (1988)]. This system is useful for sequencing a wide variety of templates such as amplified DNA, large double-stranded DNA templates such as lambda, GC-rich templates and palindrome-rich templates.

Existing thermostable polymerases which are used in chain termination methods of sequencing (both traditional and thermal cycling protocols) require fairly high concentrations of ddNTPs as the affinity of these polymerases for ddNTPs is somewhat low. For example, when *Taq* DNA polymerase is employed for chain termination sequencing protocols, the optimal concentrations of ddNTPs in the ddNTP termination mixtures (3X mixtures): 180 μM ddGTP, 1 mM ddATP, 1.5 mM ddTTP and 500 μM ddCTP [as described in U.S. Patent 5,075,216, the disclosure of which is herein incorporated by reference]. When the polymerase employed is a modified form of *Taq* DNA polymerase, sTaq (sequencing grade *Taq*), the optimal concentrations of ddNTPs in the ddNTP termination mixtures: 30 μM ddGTP, 350 μM ddATP, 600 μM ddTTP

and 200 μ M ddCTP. In contrast, a modified form of *Tne* DNA polymerase provided herein utilizes the following concentrations of ddNTPs in the termination mixtures (3X mixtures): 20 μ M ddGTP, 50 μ M ddATP, 75 μ M ddTTP and 25 μ M ddCTP. Because ddNTPs are expensive, the use of a thermostable polymerase having a higher affinity for ddNTPs (*i.e.*, the modified *Tne* polymerase of the invention) will result in considerable cost savings in DNA sequencing applications.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); g (gravitational field); vol (volume); w/v (weight to volume); v/v (volume to volume); BSA (bovine serum albumin); CTAB (cetyltrimethylammonium bromide); fmol (femtomole); HPLC (high pressure liquid chromatography); DTT (dithiothreitol); DMF (N, N dimethyl formamide); DNA (deoxyribonucleic acid); i.d. (internal diameter); p (plasmid); μ l (microliters); ml (milliliters); μ g (micrograms); pmoles (picomoles); mg (milligrams); MOPS (3-[N-Morpholino]propanesulfonic acid); M (molar); mM (milliMolar); μ M (microMolar); nm (nanometers); kdal (kilodaltons); OD (optical density); EDTA (ethylene diamine tetra-acetic acid); FITC (fluorescein isothiocyanate); SDS (sodium dodecyl sulfate); NaPO₄ (sodium phosphate); Tris (tris(hydroxymethyl)-aminomethane); PMSF (phenylmethylsulfonylfluoride); TBE (Tris-Borate-EDTA, *i.e.*, Tris buffer titrated with boric acid rather than HCl and containing EDTA) ; PBS (phosphate buffered saline); PPBS (phosphate buffered saline containing 1 mM PMSF); PAGE (polyacrylamide gel electrophoresis); Tween (polyoxyethylene-sorbitan); Boehringer Mannheim (Boehringer Mannheim,

Indianapolis, IN); Epicentre (Epicentre Technologies, Madison, WI); New England Biolabs (New England Biolabs, Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia Biotech Inc., Piscataway, NJ); Perkin Elmer (Perkin Elmer, Norwalk, CT); Promega (Promega Corp., Madison, WI); Qiagen (Qiagen Inc., Chatsworth, CA); Spectra (Spectra, Houston, TX); Stratagene (Stratagene Cloning Systems, La Jolla, CA); USB (U.S. Biochemical, Cleveland, OH).

EXAMPLE 1

Isolation Of The *Tne* DNA Polymerase Gene

a) Growth of *T. neapolitana* Cells

T. neapolitana cells (obtained from V.A. Svetlichny, The Institute of Microbiology, Russian Academy of Sciences, Moscow) were grown in a medium containing (per 100 ml): 0.1 ml K-phosphate solution [300 g/l K_2HPO_4 and 200 g/l KH_2PO_4]; 1 ml of Solution 1 [27 g/l NH_4Cl , 27 g/l $CaCl_2$ and 31 g/l $MgCl_2 \cdot 6H_2O$]; 1 ml of a 10% solution of yeast extract (Difco); 2.5% natural sea salt; 0.1 ml of a 2% solution of resazurin; 1 ml of 5% Na_2SO_3 ; 150 mg $NaHCO_3$ and 0.5 % glucose.

b) Isolation Of Genomic DNA

Large scale cultures (10 liters) of *T. neapolitana* cells were grown in the above medium in a 10 l fermentation vessel under nitrogen (*i.e.*, anaerobic conditions) at 75°C for 28 hours (early stationary phase). The cells were then collected by centrifugation at 10,000 x g and the cell pellet was washed once with a solution comprising 0.9% NaCl. The washed cell pellet was frozen at -70°C. DNA was isolated from the frozen cells as follows. The frozen cells (3 g) were thawed in 30 ml of a solution containing 100 mM Tris-HCl (pH 9.0), 50 mM EDTA and 2 mg/ml

lysozyme. The mixture was incubated for 30 min at 0°C and then SDS and proteinase K was added to a final concentration of 1% and 100 µg/ml, respectively. The mixture was incubated for 1.5 hours at 45°C with light shaking. Following the incubation, the mixture was cooled to room temperature (about 25°C) and NaCl was added to a concentration of 0.5 M. An equal volume of phenol/chloroform was added and the mixture was extracted and the aqueous and organic phases were separated by centrifugation for 10 min at 6,000 x g at room temperature. The supernatant was transferred to a fresh tube using a wide-bore pipet. A total of six phenol/chloroform extractions were performed (until the interphase disappeared). The DNA was precipitated by the addition of ethanol and gently mixing the solution. The precipitated DNA was washed with 70% ethanol. The DNA was then centrifuged for 5 min at 10,000 x g and the supernatant was discarded. The pellet was resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and stored at -20°C until used.

c) **Construction Of A *T. neapolitana* Genomic DNA⁺ Library**

The *T. neapolitana* DNA was then digested with *Sau3A* under conditions which promoted the generation of fragments 3-8 kb in length. Briefly, 10 µg of genomic DNA was digested with 1.6 units of *Sau3A* in a volume of 15 µl for 1 hour at 37°C. The reaction was stopped by the addition of 5 µl of sample buffer [70% glycerol, 50 mM EDTA] and the digested DNA was run on a low melting temperature agarose gel (BioRad). Fragments 3-8 kb in length were isolated from the gel using standard procedures [Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, NY (1989) pp. 6.30-6.31]. The DNA recovered from the gel was precipitated with ethanol, dried and resuspended in 100 µl of 10 mM

Tris-HCl (pH 7.5), 0.1 mM EDTA. Five microliters of the DNA mixture (about 0.1 μ g) was ligated to 0.25 μ g of the pTZ19R vector (Pharmacia) which had been digested with *Bam*HI and treated with bacterial alkaline phosphatase. The ligation products were used to transform competent TG1 cells [TG1 cells are an EcoK⁻ derivative of JM101 cells; a commercially available equivalents include NM522 cells (Pharmacia) and XL1-Blue cells (Stratagene)] and the cells were plated onto MacConkey agar plates (Difco).

White colonies (*i.e.*, those containing plasmids having an insert) were picked onto 30 master plates (96 colonies/plate; a total of about 3,000 colonies were screened). Replica plates were generated to provide cells for DNA polymerase analysis. The library was screened by functional assay; the cells from each replica plate were removed and pooled by rinsing the plate with 2 ml of 0.9% NaCl. The cells were then collected by centrifugation (12,000 rpm for 3 min) in a microcentrifuge (Eppendorf). The pellets were then washed with 1.5 ml of 0.9% NaCl. The washed cell pellets were then lysed by sonication in 0.5 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 5 mM PMSF. The lysates were then heated to 72°C-75°C in a water bath for 20 min. Following the incubation, the lysates were clarified by centrifugation in a microfuge at 12,000 rpm for 10 min. The supernatant was removed to a fresh tube. DNA polymerase activity was assayed using the supernatant as follows.

Denatured bovine thymus DNA was prepared as follows. A solution comprising 6 mM bovine thymus DNA (BioLAR, Olaine, Latvia; equivalent preparations of calf thymus DNA are available from Sigma, St. Louis, MO) in 1 mM NaOH was incubated for 15 min at 20°C. The solution was then neutralized by the addition of HCl to a final concentration of 100 mM and Tris-HCl, pH 8.0 to a final concentration of 50 mM.

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The following components were mixed: 2.5 μ l 0.5 M Tris-HCl (pH 7.6), 5 μ l 100 mM $MgCl_2$, 2 μ l denatured bovine thymus DNA (2 mg/ml), 0.2 μ l 12.5 mM of each of the dNTPs and 1.5 μ lCi of α - ^{32}P -dTTP and H_2O to a volume of 25 μ l. Twenty-five microliters of supernatant from each of the pools of lysed cells were mixed with 25 μ l of the above assay mixture in the well of a 96 well microtiter plate. The mixture was incubated for 1 hour at 75°C. The reaction was stopped by the addition of 5 μ l of 200 mM EDTA (pH 8.0). Five microliters of the reaction mixture was then loaded onto a 1 X 1 inch square of DEAE paper (Whatman). The samples were dried at 80°C and then washed with 0.5 M sodium phosphate (pH 7.2) (wash solution) using about 5 ml of wash solution per sample for 10 min with light shaking. Three washes were performed. The samples were then rinsed with water (10 ml/sample) for 1 min followed by an ethanol rinse. The ethanol rinsed samples were then dried at 80°C and DEAE-absorbent radioactivity was counted using a liquid scintillation counter. The results of the initial DNA polymerase assays revealed that a single pool produced DNA polymerase activity.

To isolate clones containing *The* genomic DNA encoding the DNA polymerase activity, the colonies on the positive master plate were grown as smaller pools comprising either a single row or a single column of colonies. The smaller pools of colonies were grown, lysates were prepared and DNA polymerase activity was determined as described above. A single row and a single column contained DNA polymerase activity; the intersection of this row and column identified the single individual clone containing DNA encoding DNA polymerase activity. This single colony was grown and assayed for DNA polymerase activity to confirm the presence of thermostable DNA polymerase activity. This clone was called pTen.

DNA was prepared from the pTen clone using standard techniques of molecular biology; this clone was found to contain an insert of approximately 3.5 kb. Restriction

enzyme digests were performed with a battery of enzymes to create a restriction map of the *Tne* genomic DNA insert. Subclones were generated from the positive clone and a series of nested deletions were generated using Exonuclease III and standard molecular biology techniques to facilitate DNA sequencing [Short Protocols in Molecular Biology, 2nd ed. (1992) Ausubel *et al.* Eds, John Wiley & Sons, New York, pp.7-8 to 7-16 and 7-29 to 7-37]. The DNA sequence of the insert was determined using the Sanger dideoxy sequencing method and Sequenase® (USB). The DNA sequence of the coding region for the full-length *Tne* DNA polymerase gene is listed in SEQ ID NO:1. The deduced amino acid sequence of the *Tne* DNA polymerase is listed in SEQ ID NO:2.

Analysis of the deduced amino acid sequence was performed using protein analysis software (DNASTar, Inc., Madison, WI). The open reading frame encodes a protein of 893 amino acids; the predicted molecular weight of the protein is 102,054 (however, as shown in Example below, the full-length protein migrates with an apparent molecular weight of 97,000 on SDS-PAGE gels). The predicted isoelectric point is 6.19 and the charge at pH 7.0 is -7.56.

The nucleotide and amino acid sequences of the *Tne* DNA polymerase were compared with the reported sequences for *E. coli* DNA polymerase I and the thermostable DNA polymerase from *T. maritima*. Figure 3 provides an alignment of the amino acid residues of these three polymerases. In Figure 3 the following abbreviations are used: Eco (*E. coli* DNA polymerase I); Tma (*Tma* DNA polymerase) and Tne (*Tne* DNA polymerase). Shading is used to indicate residues which differ from the amino acid sequence of *Tne* DNA polymerase.

In *E. coli* DNA polymerase I, the 5' exonuclease domain comprises approximately residues 1-323; the 3' exonuclease domain comprises approximately residues 324-517 and the synthetic or polymerization domain comprises approximately

residues 521-928. Alignment of the amino acid sequences of *E. coli* DNA polymerase I with the sequence of the *Tne* DNA polymerase molecule of the present invention reveals that the 5' exonuclease domain of *Tne* DNA polymerase comprises approximately residues 1-297; the 3' exonuclease domain comprises approximately residues 298-482 and the polymerization domain comprises approximately residues 486-893.

Alignment of amino acid residues present in *E. coli* DNA polymerase I and *Tne* DNA polymerase shows that the two enzymes are 44% identical overall and 51% identical over the polymerase domain (residues 521-928 in *E. coli* and residues 486-893 in *Tne*). The alignment was performed using the Lipman-Pearson algorithm as provided by DNASTAR, Inc. (Madison, WI); gaps were introduced into the two sequences to provide for maximum alignment.

Comparison of the nucleotide and amino acid sequences of the *Tne* and *Tma* polymerases revealed that these two polymerases share 78% identity at the nucleotide level and 88% identity at the amino acid level.

EXAMPLE 2

Efficient Expression Of *Tne* DNA Polymerase In *E. coli*

In order to express the *Tne* DNA polymerase in large amounts in host cells, the DNA sequences encoding the polymerase (*i.e.*, the coding region) were removed from pTen (described in Example 1) and inserted into an expression vector.

Restriction enzyme analysis revealed that the *Tne* polymerase gene was present in the opposite transcriptional orientation relative to the T7 promoter present on the pTZ19R vector used to generate pTen. In order to produce *Tne* polymerase, sequences encoding the *Tne* polymerase gene were removed from pTen by digestion with *Sma*I

and *Xba*I and an approximately 3.5 kb *Sma*I/*Xba*I fragment was isolated by electrophoresis of the digestion products on an agarose gel followed by excision of the desired band. DNA was recovered from the agarose block using the Wizard™ PCR Preps DNA Purification System (Promega). Briefly, 0.5 to 1.0 ml of Wizard™ PCR Preps DNA Purification Resin was added to the agarose block and the mixture was incubated at 42°C for 5 minutes to melt the agarose. DNA was extracted using the protocol provided in the kit.

The 3.5 kb *Sma*I/*Xba*I fragment was ligated into the pGEM®-3Zf(+) vector (Promega) which had been digested with *Sma*I and *Xba*I to generate pGTne. This construction placed the 5' end (*i.e.*, encodes the N terminal portion) of the *Tne* polymerase gene downstream of the T7 promoter in the same transcriptional orientation. The ligation mixture was used to transform competent JM109(DE3) cells (Promega). Recombinant clones were isolated, confirmed by restriction digestion using standard recombinant molecular biology techniques [Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989)].

Expression of the *Tne* DNA polymerase from the pGTne construct was next examined. JM109(DE3) cells containing pGTne were grown at 37°C and induced with 1 mM IPTG. Parallel cultures of TG1 cells containing pTen were grown and induced. After a few hours (*i.e.*, 1-3) of growth in the presence of IPTG, the cells were collected by centrifugation and crude lysates were prepared as follows. A 1 ml aliquot of each of the cultures containing the *Tne* constructs was centrifuged in a microcentrifuge at 14,000 x *g* for 3 min at room temperature to pellet the cells. The cells were then resuspended in 200 µl of a solution comprising 50 mM Tris-HCl (pH 8.0), 50 mM glucose, 1 mM EDTA and the cells were pelleted again. The cells were next resuspended in 50 µl of the previous buffer containing 4 mg/ml lysozyme and the mixture was incubated at room temperature for 10 minutes. Following the incubation,

50 μ l of a solution comprising 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.5% Tween-20, 0.5% Nonidet P40 was added and the mixture was incubated at 75°C for 10 minutes. The lysate was then clarified by centrifugation in a microcentrifuge at 14,000 x g for 5 minutes. Eighty microliters of the supernatant was removed and stored in a separate tube at 4°C. The crude lysates were analyzed for polymerase activity at 74°C as described in Example 5(b), below.

The following results were obtained. The pTen construct gave polymerase activity levels at or about 2 fold higher than the background level for the assay. The pGTne construct gave activity levels of about 50 times background levels. While the expression of *Tne* polymerase seen using pGTne was much improved relative to the level seen using pTen, this expression level was not sufficient to produce large amounts of the enzyme.

These above result suggested that the *Tne* polymerase promoter was non-functional in *E. coli* (very low level of activity present when pTen is used). Furthermore, the presence of the *Tne* polymerase promoter appeared to be detrimental to expression when transcription was initiated from the T7 promoter in the pGTne construct (perhaps due to transcriptional read-through interference). In order to remove the *Tne* polymerase gene promoter from the *Tne* polymerase coding region, the following experiments were conducted.

The DNA sequence of the 5' end of the *Tne* polymerase gene was sequenced using the M13 forward primer in conjunction with the fmol[®] DNA Sequencing System (Promega); sequencing was conducted according to the manufacturer's instructions. The sequence analysis revealed that a unique *Bgl*II site was found 43 bp into the coding region (*i.e.*, 43 bp following the A of the initiator ATG codon). To remove the coding region of the *Tne* polymerase gene from pGTne, pGTne was digested with *Bgl*II and *Xba*I and the approximately 3.0 kb *Bgl*II/*Xba*I fragment was isolated (as described

above). The 3.0 kb *Bgl*II/*Xba*I fragment was ligated directly downstream of either the T7 promoter or the *tac* promoter present in pALTER®-Ex1 (Promega); pALTER®-Ex1 contains both the T7 and the *tac* promoters positioned in opposite transcriptional orientations relative to one another. These two ligations were performed as follows.

To insert the 3.0 kb *Bgl*II/*Xba*I fragment downstream of the T7 promoter, a 43 bp synthetic linker having a *Nco*I overhanging end at one end and a *Bgl*II overhanging end at the other end was ligated to the *Tne* polymerase coding region. This linker was formed by annealing of the following two oligonucleotides: JH64 which comprises 5'-CATGGCGAGACTATTTCTCTTTGATGGCACAGCCCTGGCCTACA-3' (SEQ ID NO:3) and JH65 which comprises 5'-AGGCCAGGGCTGTGCCATCAAAGAGAA ATAGTCTCGC-3' (SEQ ID NO:4). This synthetic linker regenerates the native sequence of the *Tne* polymerase gene located upstream of the *Bgl*II site and allows insertion of the coding region into pALTER®-Ex1. pALTER®-Ex1 was digested with *Nco*I and *Xba*I and the coding region containing the synthetic linker was ligated to the digested vector to generate pATne2.

To insert the 3.0 kb *Bgl*II/*Xba*I fragment downstream of the *tac* promoter, a 43 bp synthetic linker having a *Nde*I overhanging end at one end and a *Bgl*II overhanging end at the other end was ligated to the *Tne* polymerase coding region. This linker is formed by annealing of the following two oligonucleotides: JH62 which comprises 5-AGGCCAGGGCTGTGCCATCAAAGAGAGAAATAGTCTCGCCA (SEQ ID NO:5) and JH63 which comprises 5'-TATGGCGAGACTATTTCTCTTTGTGGCACAGCCCT GGCCTACA-3' (SEQ ID NO:6). This synthetic linker regenerates the native sequence of the *Tne* polymerase gene located upstream of the *Bgl*II site and allows insertion of the coding region into pALTER®-Ex1. pALTER®-Ex1 was digested with

*Nde*I and *Xba*I and the coding region containing the synthetic linker was ligated to the digested vector to generate pATne1.

Competent *E. coli* cells were transformed with the above ligation mixtures corresponding to either pATne1 (JM109 cells; Promega) and pATne2 [JM109(DE3) cells; Promega]. Recombinant clones were isolated, confirmed by restriction digestion using standard recombinant molecular biology. Cells harboring either pATne1 or pATne2 were grown and induced as described above. Crude lysates were prepared and DNA polymerase assays were performed (as described above). The results of these polymerase assays showed that both pATne1 and pATne2 gave significantly better yields of *Tne* polymerase than pGTne (at least 2-3 fold higher).

EXAMPLE 3

Construction Of *Tne* Deletion Mutants Lacking 5' To 3' Exonuclease Activity

As noted above, the presence of 5' to 3' exonuclease activity in a thermostable DNA polymerase is undesirable for certain applications. To construct mutant *Tne* polymerases lacking 5' to 3' exonuclease activity, two deletion mutants of the *Tne* polymerase gene were generated. Both mutants contain deletions which remove sequences encoding a large portion of the 5' to 3' exonuclease domain located at the N terminus of the *Tne* polymerase molecule.

a) Construction Of A Vector Encoding Deletion Mutant Tne M284

The deletion mutant Tne M284 is a truncated form of the *Tne* polymerase which uses the naturally occurring methionine at amino acid position 284 in the full-length protein (SEQ ID NO:2) as the initiating methionine for translation

initiation. Figure 4 provides a schematic representation of several modified *Tne* polymerases (constructed as described in Examples 3 and 4) along the map of the full-length *Tne* polymerase protein. The scale represents length in increments of 100 amino acid residues. The full length *Tne* polymerase (SEQ ID NO:2) contains 893 amino acids. The thick open boxes represent the presence of amino acid residues; thin lines between two regions of thick boxes indicates that amino acids were deleted between the two open boxes. Circles containing a single letter indicate the location of a mutated amino acid residue (the single letter code is used for the amino acid residues indicated).

To generate a construct containing the *Tne* M284 mutant, pGTne was digested with *Bsp*HI (generates ends compatible with *Nco*I ends) and *Kpn*I and a 1.05 kb *Bsp*HI/*Kpn*I fragment (containing the 5' portion of the coding region) was isolated as described in Example 2. A second aliquot of pGTne was digested with *Kpn*I and *Xba*I and a 1.3 kb fragment containing the 3' portion of the *Tne* polymerase coding region was isolated. pALTER-Ex1 was digested with *Nco*I and *Xba*I. A three-way ligation was performed using the digested pALTEREx1 vector, 1.05 kb *Bsp*HI/*Kpn*I fragment and the 1.3 kb *Kpn*I/*Xba*I fragment. Competent JM109(DE3) cells were transformed with the ligation mixture and recombinant clones were isolated, confirmed by restriction digestion using standard recombinant molecular biology. The resulting plasmid was called pM284. The nucleotide sequence of the region encoding the *Tne* M284 gene is listed in SEQ ID NO:7. The amino acid sequence of *Tne* M284 is listed in SEQ ID NO:8.

**b) Construction Of A Vector Encoding Deletion Mutant
Tne M316**

The deletion mutant Tne M316 is a truncated form of the *Tne* polymerase protein which uses an artificially created methionine at amino acid position 316 as the initiator methionine. Tne M316 was created by introducing a methionine residue at position 316 (and a corresponding *NcoI* site) via site-directed mutagenesis using the Altered Sites® II *in vitro* Mutagenesis System (Promega) in conjunction with mutagenesis oligonucleotide JH68 [5'-ATCGAAAAGCTGACCATGGTTCCATCTT TTG-3' (SEQ ID NO:9)] and pATne2. The manufacturer's protocol was followed exactly. Briefly, pATne2 was denatured using alkali and the JH68 mutagenic oligonucleotide was annealed to the denatured plasmid along with the ampicillin repair oligonucleotide (provided in the kit). The mutant strand was synthesized using T4 DNA polymerase and T4 DNA ligase. ES1301*mutS* cells (provided in the kit) were then cotransformed with the mutagenized pATne2 and R408 DNA (provided in the kit). Small scale DNA preparations were prepared from the transformed ES1301*mutS* cells and the DNA was used to transform JM109 cells. Mutants were selected by growth on ampicillin plates and the desired recombinant were confirmed by restriction enzyme analysis (*i.e.*, presence of an additional *NcoI* site). The resulting plasmid containing the engineered *NcoI* site was then digested with *NcoI* which deleted the 5' to 3' exonuclease domain as a 948 bp fragment and the large fragment was isolated (as described above) and religated to itself to create the pM316 construct. The nucleotide sequence of the region encoding the Tne M316 gene is listed in SEQ ID NO:10. The amino acid sequence of Tne M316 is listed in SEQ ID NO:11.

c) Expression Of Tne M284 And Tne M316 In *E. coli*

The pM284 and pM316 constructs (in JM109 cells) were grown, induced and crude lysates were prepared as described in Example 2. DNA polymerase activity was measured in crude lysates as described in Example 2.

5 The results of the polymerase assays showed that the Tne M284 mutant (pM284) contained 17 units per μ l of polymerase activity from the crude lysate and the Tne M316 mutant (pM316) produced no detectable polymerase activity. No detectable polymerase activity was found when the Tne M316 polymerase was expressed from the *trc* promoter either (to express the Tne M316 coding region from the *trc* promoter, a 2.5 kb *NcoI/PstI* fragment was isolated from pM316 and ligated to pTrc 99 A (Pharmacia) digested with *NcoI* and *PstI*).

10 Aliquots (5 μ l) of each crude lysate were electrophoresed on a pre-cast 4-20 % denaturing gradient polyacrylamide gel (Novex, San Diego, CA); following electrophoresis, the gel was stained with Coomassie blue to visualize the separated proteins. A single, sharp protein band corresponding to the expected size was visible in lysates produced from cells containing the pATne1 (full-length *Tne* polymerase) and pM284 (Tne M284 deletion mutant) constructs. No protein band was observed for deletion mutant Tne M316 when expressed from either the T7 or *trc* promoters.

15 In order to increase the level of expression of Tne M284 protein in *E. coli*, the Tne M284 coding region was placed downstream of the strong *tac* promoter present in the JHEX3 vector to create pJM284. JHEX3 was created as follows. pALTER-1 (Promega) was digested with *ClaI* and *SlyI* and the ends were made blunt by incubation with the Klenow fragment. The 1.345 kb *ClaI/SlyI* fragment was isolated and ligated into pTrc 99 A (Pharmacia) which had been digested with *BsaAI*. This
20 ligation inserted the tetracycline-resistance gene into the pTrc 99 A vector; the resulting vector was called JHEXa. The ampicillin-resistance gene was then removed
25

from the JHEXa by digestion with *SspI*, *DraI* and *PvuI*; this digestion cut the
ampicillin gene into four small fragments (483 bp, 227 bp, 209 bp and 19 bp). The
large fragments (3.93 kb and 652 bp) were isolated and ligated together to create
JHEXb. The *Trc* promoter was removed from JHEXb as an 89 bp *SspI/NcoI* fragment
and replaced with the *tac* promoter. The *tac* promoter was inserted into the
SspI/NcoI-digested JHEXb vector as a 141 bp *BsrBI* fragment from pALTER-Ex1
(Promega) together with a 30 bp linker formed by the oligonucleotide pair listed in
SEQ ID NOS:45 and 46.

To generate pJM284 construct was made as follows. pGTne was digested with
BspHI (generates ends compatible with *NcoI* ends) and *KpnI* and a 1.05 kb
BspHI/KpnI fragment (containing the 5' portion of the coding region) was isolated as
described in Example 2. A second aliquot of pGTne was digested with *KpnI* and *XbaI*
and a 1.3 kb fragment containing the 3' portion of the *Tne* polymerase coding region
was isolated. JHEX3 was digested with *NcoI* and *XbaI*. A three-way ligation was
performed using the digested JHEX3 vector, 1.05 kb *BspHI/KpnI* fragment and the 1.3
kb *KpnI/XbaI* fragment. Competent JM109 cells were transformed with the ligation
mixture and recombinant clones were isolated, confirmed by restriction digestion using
standard recombinant molecular biology. The resulting plasmid was called pJM284.

Crude lysates were prepared from a small scale culture of JM109 cells
containing the pJM284 construct or the pM284 construct. DNA polymerase assays
were performed as described in Example 2. The level of Tne M284 polymerase
produced by pJM284 was found to be about 50% greater than the level produced by
expression from the pM284 construct.

EXAMPLE 4

Construction Of *Tne* Polymerase Mutants Having Altered 3' To 5' Exonuclease Activity

In order to produce modified forms of *Tne* polymerase which possess varying amounts of 3' to 5' exonuclease activity, seven different point mutants and two deletion mutants were created using the pM284 construct as the starting material. Figure 4 provides a schematic drawing of these mutant *Tne* polymerases.

All nine mutagenic changes also involved a change in the restriction digest pattern of the starting pM284 plasmid to allow for easy selection of the mutants. In all cases, a small portion of the mutagenized region was exchanged into an *Tne* M284 gene that did not undergo mutagenesis and the exchanged region was sequenced not only to confirm the mutation, but also to show that there were no second site mutations. DNA sequencing was performed using the fmol® DNA Sequencing System (Promega) in conjunction with using primers JH61 [5'-TGCCGTACACCTCC GAGAGC-3' (SEQ ID NO:12)] or JH66 [5'-CTCGTTTGGCTCCAGCAAATATGC-3' (SEQ ID NO:13)]. The mutants were constructed as follows.

a) Construction Of pD323E

pD323E produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 323 (number indicates position of the residue in the full length protein). At amino acid residue 323 the wild-type aspartic acid is replaced with glutamic acid. pM284 was used in conjunction with the mutagenic oligonucleotide JH74 [5'-TTTGCCCTGGAaCTTGAAACG-3' (SEQ ID NO:14)]; the mutagenic residues are indicated by the lower case letter] and the Altered Sites® II *in vitro*

Mutagenesis System (Promega) to generate pD323E as described in Example 3. The desired mutants were confirmed by restriction analysis (absence of one of the *SinI* restriction sites present in pM284. The DNA sequence of pD323E was obtained as described above using the JH66 (SEQ ID NO:13) primer. The DNA sequence of the polymerase coding region present in pD323E is listed in SEQ ID NO:15. The corresponding amino acid sequence of the Tne M284(D323E) protein is listed in SEQ ID NO:16.

b) Construction Of pE325D

pE325D produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 325. At amino acid residue 325, the wild-type glutamic acid residue is replaced with aspartic acid. pM284 was used in conjunction with the mutagenesis oligonucleotide JH75 [5'-GACCTTGAcACGTCCTC (SEQ ID NO:17);the mutagenic residue is indicated by the lower case letter] and the Altered Sites® II *in vitro* Mutagenesis System (Promega) to generate pE325D as described in Example 3. The desired mutant was confirmed by restriction analysis (the presence of additional *AflIII* restriction site). The DNA sequence of pD323 was obtained as described above using the JH66 (SEQ ID NO:13) primer. The DNA sequence of the polymerase coding region present in pE325D is listed in SEQ ID NO:18. The corresponding amino acid sequence of the Tne M284(E325D) protein is listed in SEQ ID NO:19.

c) Construction Of pY464F

pY464F produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 464. At amino acid residue 464, the wild-type tyrosine

residue is replaced with phenylalanine. pY464F was constructed by replacing a 39 bp *FokI* fragment present in pM284 with a 39 bp synthetic region. The 39 bp synthetic region was formed by the following two oligonucleotides: JH81 [5'-TAAGTGATATC TGCATCCTCGCAGGAGAAGTTCGCAGCC-3' (SEQ ID NO:20) and JH82 [5'-ACAAGGCTGCGAACTTCTCCTGCGAGGATGCAGATATCA-3' (SEQ ID NO:21)]. This synthetic 39 bp oligonucleotide contains the mutation. The desired mutant was confirmed by restriction analysis (the presence of additional *EcoRV* restriction site). The DNA sequence of pY464F was obtained as described above using the JH61 (SEQ ID NO:12) primer. The DNA sequence of the polymerase coding region present in pY464F is listed in SEQ ID NO:22. The corresponding amino acid sequence of the Tne M284(Y464F) protein is listed in SEQ ID NO:23.

d) Construction Of pD468N

pD468N produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 468. At amino acid residue 468, the wild-type aspartic acid residue is replaced with asparagine. pM284 was used in conjunction with the mutagenesis oligonucleotide JH79 [5'-ACTCCTGCGAGaATGCtGACATCACTTAT AGG-3' (SEQ ID NO:24); the mutagenic residues are indicated by the use of lower case letters] and the Altered Sites® II *in vitro* Mutagenesis System (Promega) to generate pD468N as described in Example 3. The desired mutant was confirmed by restriction analysis (the presence of an additional *BsmI* restriction site). The DNA sequence of pD468N was obtained as described above using the JH61 (SEQ ID NO:12) primer. The DNA sequence of the polymerase coding region present in pD468N is listed in SEQ ID NO:25. The corresponding amino acid sequence of the Tne M284(D468N) protein is listed in SEQ ID NO:26.

e) **Construction Of pD323A**

pD323A produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 323. At amino acid residue 323, the wild-type aspartic acid residue is replaced with alanine. pM284 was used in conjunction with the mutagenesis oligonucleotide JH70 [5'-TTTGCCCTGGcCCTTGAAACG-3' (SEQ ID NO:27); the mutagenic residue is indicated by the use of the lower case letter] and the Altered Sites® II *in vitro* Mutagenesis System (Promega) to generate pD323A as described in Example 3. The desired mutant was confirmed by restriction analysis (the absence of a *SinI* restriction site). The DNA sequence of pD323A was obtained as described above using the JH66 (SEQ ID NO:13) primer. The DNA sequence of the polymerase coding region present in pD323A is listed in SEQ ID NO:28. The corresponding amino acid sequence of the *Tne* M284(D323A) protein is listed in SEQ ID NO:29.

f) **Construction Of pD389A**

pD389A produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains an amino acid substitution at residue 389. At amino acid residue 389, the wild-type aspartic acid residue is replaced with alanine.

To construct pD389A, the PCR was used to amplify two overlapping fragments independently; the PCR products were combined and the resulting large fragment was reamplified. Mutagenesis oligonucleotide JH80 [5'-CCTGAAGTACGcgTACAAGGT TCTTATGG-3' (SEQ ID NO:30); the mutagenic residues are indicated by the use of lower case letters] and sequencing primer JH61(SEQ ID NO:12) were used to prime a first PCR to create a 425 bp fragment which incorporates the desired mutation. The sequencing primers JH66 (SEQ ID NO:13) and M13 reverse (Promega Q5401; SEQ

1004319-031000
5 ID NO:31) were used to amplify a 564 bp fragment using pM284 as the template in a second PCR. When these two PCR products were combined using the M13 reverse and JH61 primers, a 889 bp fragment was made. A 348 bp *Bgl*II fragment was then removed from the 889 bp product and was exchanged with the analogous, but, non-mutagenic *Bgl*II fragment of pM284. The desired mutants was confirmed by the presence of an extra *Mlu*I restriction site. All PCR reactions were performed using a Perkin-Elmer 480 thermal cycler.

10 For generation of the 564 bp product, the PCR was conducted by performing 15 cycles comprising a denaturation step (95°C for 15 sec) and an annealing/extension step (70°C for 1 min). JM284 was used as the template in a reaction containing 1 μ M of each of the primers (JH66 and M13 reverse), 1.5 mM MgCl₂ and 3 units *Tli* DNA polymerase (Promega).

15 For generation of the 425 bp product, the PCR was conducted by performing 20 cycles comprising a denaturation step (95°C for 15 sec), an annealing step (55°C for 30 sec; cycles 1-5) or an annealing step (70°C for 15 sec; cycles 6-20) and an extension step (70°C for 45 sec). JM284 was used as the template in a reaction containing 1 μ M of each of the primers (JH80 and JH61), 1.5 mM MgCl₂ and 3 units *Tli* DNA polymerase (Promega).

20 For the generation of the 889 bp product, the 564 bp product and the 425 bp product were used as the template in a reaction containing 1.5 mM MgCl₂ and 3 units *Tli* DNA polymerase (Promega). The cycling conditions were: denaturation (95°C for 15 sec) and annealing/extension at 70°C for 1 min; no primers were present for cycles 1-5. One μ M of the M13 reverse primer was present for cycles 5-10 and 1 μ M of the M13 reverse and JH61 primers were present in cycles 11-25.

25 The DNA sequence of pD389A was obtained as described above using the JH61 and JH66 (SEQ ID NOS:12 and 13) primer. The DNA sequence of the

polymerase coding region present in pD389A is listed in SEQ ID NO:32. The corresponding amino acid sequence of the Tne M284(D389A) protein is listed in SEQ ID NO:33.

g) Construction Of pD323,389A

pD323,389A produces a modified form of the *Tne* polymerase which lacks the first 283 amino acids from the N-terminus of the full-length protein and contains two amino acid substitutions at residues 323 and 389. At amino acid residue 323, the wild-type aspartic acid residue is replaced with alanine and at amino acid residue 389, the wild-type aspartic acid residue is replaced with alanine.

To create pD323,389A, the 215 bp *Csp45I* fragment of pD323A was exchanged with the analogous fragment in pD389A bringing the two single mutations into the same construct. The desired mutants were selected as having the two restriction site changes of the individual mutants (described above). The DNA sequence of pD323,389A was obtained as described above using the JH66 (SEQ ID NO:13) primer. The DNA sequence of the polymerase coding region present in pD323,389A is listed in SEQ ID NO:34. The corresponding amino acid sequence of the Tne M284(D323A,D389A) protein is listed in SEQ ID NO:35; this enzyme is referred to as the triple mutant *Tne* polymerase.

When all of the above point mutant constructs (sections a-g) were induced to express the modified *Tne* polymerases in an *E. coli* host, the crude extracts showed polymerase activity comparable with the activity observed using the original pM284 construct (cultures were grown, induced, lysates prepared and assayed for DNA polymerase activity as described in Example 2).

h) **Construction Of Deletion Mutants pM323 And pJM284ΔB**

Two mutants were created which contained deletions into the putative 3' to 5' exonuclease domain of the *Tne* polymerase gene. It was predicted that these two deletion mutations would remove all 3' to 5' exonuclease activity. Surprisingly, when these two mutants were expressed in *E. coli* no polymerase activity was detected.

i) **Construction Of pM323**

pM323 was constructed by digestion of pJM284 with *SinI* and *EcoRV* followed by removal of the 3' overhanging (*i.e.*, sticky) ends with Mung Bean nuclease. The 1.9 kp *SinI/EcoRV*(polished ends) fragment was isolated as described in Example 2. JHEX3 (Example 3) was digested with *NcoI* and *SmaI* and the *NcoI* overhanging ends were made blunt by incubation with the Klenow fragment. The 1.9 kp *SinI/EcoRV*(polished ends) fragment was then blunt end ligated into the prepared JHEX3 vector. When the blunted *SinI* end ligates to the blunted *NcoI* end 39 amino acids are removed from the N terminus of the protein encoded by the pM284 construct; this junction also creates an initiating methionine at amino acid position 323 that is in-frame with the rest of the coding region. The polymerase coding region present in the pM323 construct was sequenced to confirm that no undesirable mutations were introduced (*i.e.*, insertion of stop codons, frame-shift mutations). The DNA sequence of the polymerase coding region present in pM323 is listed in SEQ ID NO:36. The corresponding amino acid sequence of the Tne M323 protein is listed in SEQ ID NO:37.

ii) Construction Of pJM284ΔB

pJM284ΔB was constructed by digestion of the JM284 construct with *Bgl*II followed by religation of the large fragment (6.6 kb) back on itself. Digestion of JM284 with *Bgl*II created two fragments, the smaller being 348 bp which contains DNA sequences which encode a portion of the 3' to 5' exonuclease domain. The pJM284ΔB construct removed 116 amino acids of the 3' to 5' exonuclease domain which correspond to residues 309 through 424 in SEQ ID NO:2. pJM284ΔB contains the same 25 amino acids which encode the N terminus of the protein encoded by pM284. This deletion does not change the reading frame for the polymerase domain. Restriction digest analysis was conducted on the pJM284ΔB construct to confirm the proper construction was made. The DNA sequence of the polymerase coding region present in pJM284ΔB is listed in SEQ ID NO:38. The corresponding amino acid sequence of the Tne M284ΔB protein is listed in SEQ ID NO:39.

iii) Expression Of pM323 And pJM284ΔB In *E. coli*

When both of these deletion mutants constructs were grown and induced to express the *Tne* polymerase in an *E. coli* host (JM109), no detectable polymerase activity was detected. As these two constructs were analyzed by restriction digestion or DNA sequencing to insure that no undesirable mutations were introduced, it appears, surprisingly, that deletion into the putative 3' exonuclease domain (approximately aa residues 291-484) is deleterious either for polymerase activity or alternatively for protein stability. These results, in conjunction with those obtained using the pM316 construct, show that deletions beyond about position 849 of SEQ ID NO:1 produce proteins which either are unstable (perhaps due to improper folding) or lack polymerase activity.

EXAMPLE 5

Purification Of Tne DNA Polymerases

In order to produce purified preparations of the wild-type and modified *Tne* polymerases, cells harboring the *Tne* expression vectors described above were grown, induced and the *Tne* polymerases were isolated.

a) Growth Of *E. coli* Cells Harboring Recombinant Tne (rTne) Constructs

E. coli strains containing a desired construct were streaked onto LB plates [10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1 ml/l 1N NaOH and 15 g/l agar] containing 10 μ g/ml tetracycline to isolate single colonies and the plates were grown overnight at 37°C. A single colony was inoculated into 150 ml LB broth [10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl and 1 ml/l 1N NaOH] containing 10 μ g/ml tetracycline (divided into three flasks of 50 ml each); the three flasks were grown with shaking overnight at 37°C. The next day, 120 ml of the overnight culture was used to seed the fermentation of 6 liters of LB containing 10 μ g/ml tetracycline prewarmed to 37°C (divided into six flasks of 1 liter each). The large scale culture was grown for 5 hours at 37°C and then IPTG was added to a final concentration of 1 mM and growth was continued for an additional 2 hours at 37°C. The induced cells were harvested by centrifugation at 9,000 rpm for 5 minutes in a Beckmann JA10 rotor. Yields were typically 2 g cell paste per liter of fermented culture.

b) Purification Of r*Tne* DNA Polymerases

Ten grams of cell paste (prepared as described above) were resuspended in 100 ml of an ice-cold solution containing 0.25 M NaCl in TEDGT buffer [50 mM Tris-HCl (pH 7.3), 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.1% Tween 20] containing 2.5 mM PMSF. The resuspended cells were lysed by sonication using a Vibracell sonicator (Model VCX600; Sonics and Materials, Inc., Danbury, CN). The solution was kept ice-cold during sonication by placement of the beaker containing the cell suspension in a salted ice bath. Sonication was repeated ten times at 40% output for 1 minute with a 2 minute rest between the 1 minute sonication bursts. The cell lysate was heat treated to denature the bulk of *E. coli* proteins by incubation of the lysate at 68°C to 70°C for 5 to 10 minutes; following heat treatment the lysate was placed on ice.

The following purification steps were performed at 4°C. The chilled lysate was centrifuged at 15,000 rpm for 15 minutes in a Beckman JA18 rotor to remove the heat-denatured proteins. The cleared lysate supernatant was removed and 4 ml of 5% polyethylenimine (PEI) was added to the supernatant to precipitate any DNA present in the lysate. The lysate was centrifuged at 15,000 rpm for 15 minutes in a Beckman JA18 rotor to remove the precipitated DNA. The supernatant was retrieved and solid ammonium sulfate was added to 60% saturation to precipitate the DNA polymerase. After dissolution of the ammonium sulfate, the sample was centrifuged at 15,000 rpm for 1 hour in a Beckman JA18 rotor. The supernatant was discarded and the precipitated proteins were gathered and dissolved in 10 ml TEDGT buffer. The resolubilized protein was then placed in a dialysis membrane tubing having a 12,000 to 14,000 mw cutoff (Spectra, Houston, TX) and then dialyzed against TEDGT buffer to remove the ammonium sulfate.

The dialyzed protein solution was then loaded onto a 15 ml Heparin Sepharose (Scientific Protein Laboratory, Waunakee, WI) column (1.7 cm i.d. x 6.5 cm height). The column was washed with 150 ml 0.05 M NaCl in TEDGT buffer. A 100 ml salt (NaCl) gradient was run over the column to elute the DNA polymerase; the gradient started at 0.05 M NaCl and ended at 1 M NaCl (all in TEDGT buffer). Fractions (1.5 ml) were collected and assayed for DNA polymerase activity at 74°C as described below.

Thermostable DNA polymerase activity was assayed by incorporation of radiolabeled dTTP into nicked and gapped (*i.e.*, activated) calf thymus DNA (prepared as described below). One unit of thermostable DNA polymerase is defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of dNTP into an acid-insoluble form in 30 minutes at 74°C. The reaction conditions comprised: 50 mM Tris-HCl (pH 9.0 at 25°C), 50 mM NaCl, 10 mM MgCl₂, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP and 5 μCi ³H-dTTP (Amersham) and 60 μg activated calf thymus DNA in a 250 μl final volume.

The reaction components were assembled at room temperature. Samples suspected of containing polymerase activity were added (5 μl containing 0.05 to 0.5 units) and the tube was incubated at 74°C; aliquots (50 μl) were withdrawn at 6, 9, 12 and 15 minutes and placed immediately into 1.5 ml microcentrifuge tubes containing 0.5 ml of ice-cold 10% TCA on ice. After 10-30 minutes on ice, the entire TCA precipitation was filtered through a GF/A filter (Whatmann). The reaction tubes were rinsed with 3 volumes of cold 5% TCA and the filters were washed twice with 10 ml of ice-cold 5% TCA followed by a rinse with 1 ml of acetone. The filters were dried and the radioactivity bound to filters was counted in a scintillation counter.

Total and background counts were determined as follows. For total cpm, 10 μl of the reaction mix (without any polymerase added) was spotted onto duplicate GF/A

filters and counted. For background counts, 50 μ l of the reaction mix (without any polymerase added) was added to duplicate tubes containing 0.5 ml cold 10% TCA and the samples were filtered through GF/A filters and washed as described above.

Activated calf thymus DNA was prepared by dissolving 1 g calf thymus DNA (#D-151, Sigma, St. Louis, MO) in 400 ml TM buffer [10 mM Tris-HCl (pH 7.3), 5 mM $MgCl_2$]. Four hundred microliters of a solution containing 40 units of RQ1-DNAse (Promega) in TM buffer was added to the DNA solution and incubated at 37°C for 10 minutes. The DNAse digestion was stopped by heating the DNA solution at 68°C for 30 minutes. The activated calf thymus DNA was stored at -20°C until used. The activated calf thymus DNA was heated to 74°C for 10 minutes and then cooled to room temperature before use.

As shown in Table 2 below, the *The* DNA polymerases generally eluted from the Heparin Sepharose column at a salt concentration of between 0.14 M and 0.29 M NaCl. Fractions containing the polymerase activity were pooled, placed into dialysis membrane tubing (as described above) and dialyzed against TEDGT buffer until the salt concentration was less than or equal to 0.05 M NaCl as measured by conductivity using a conductance meter (Yellow Spring Instrument Co., Yellow Springs, OH).

The dialyzed polymerase fraction was then loaded onto a 7 ml Cibracron Blue 3GA (Sigma, St. Louis, MO) column (1.25 cm i.d. x 6 cm height). The Cibracron Blue 3GA column was washed with 70 ml of 0.05 M NaCl in TEDGT buffer. A 100 ml salt gradient was run over the column to elute the DNA polymerase; the gradient started at 0.05 M NaCl and ended at 1 M NaCl (all in TEDGT). Fractions (1.5 ml) were collected and assayed for DNA polymerase activity at 74°C (as described above).

As shown in Table 2, the *The* DNA polymerases generally eluted from the Cibracron Blue 3GA column at a salt concentration of between 0.20 M and 0.46 M NaCl. Fractions containing the polymerase activity were pooled, placed in dialysis

membrane (as described above) and dialyzed against storage buffer [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol and 0.5% Tween 20]. Aliquots of preparations of purified *Tne* polymerases were electrophoresed on SDS-PAGE gels and stained with Coomassie blue. The purified DNA polymerases produced by all of the recombinant *Tne* polymerase constructs were judged to be at least 95% pure based on visual inspection of the Coomassie-stained SDS PAGE gels.

TABLE 2

Salt Elution Characteristics For *rTne* DNA Polymerases

	SEQ ID NO	Heparin Sepharose	Cibacron Blue
rTne DNA Polymerase full-length	2	0.26 to 0.37 M NaCl	0.5 to 0.8 M NaCl
Tne M284	8	0.13 to 0.3 M NaCl	0.05 to 0.3 M NaCl
Tne M284 (E325D)	19	0.15 to 0.28 M NaCl	0.3 to 0.48 M NaCl
Tne M284 (D468N)	26	0.11 to 0.23 M NaCl	0.16 to 0.35 M NaCl
Tne M284 (D323E)	16	0.05 to 0.32 M NaCl	0.11 to 0.4 M NaCl
Tne M284 (Y464F)	23	0.11 to 0.28 M NaCl	0.24 to 0.4 M NaCl
Tne M284 (D323A, D389A)	35	0.14 to 0.27 M NaCl	0.06 to 0.46 M NaCl

The above results provide methods for the isolation of the full-length and modified forms of Tne DNA polymerase in a highly pure form at high yields.

EXAMPLE 6

Purification Of *The* DNA Polymerase From *T. neapolitana* Cells

The preceding example described the isolation of recombinant *The* DNA polymerases from *E. coli* harboring plasmids which overexpress these enzymes. The full-length *The* DNA polymerase may also be isolated from *T. neapolitana* cells

T. neapolitana cells are obtained from the ATCC (ATCC 49049) and grown in anaerobic culture in MMS medium containing (per liter): 6.93 g NaCl; 1.75 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.38 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 0.16 g KCl; 25 mg NaBr; 7.5 mg H_3BO_3 ; 3.8 mg $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$; 0.025 mg KI; 0.38 g CaCl_2 ; 0.5 g KH_2PO_4 ; 0.5 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$; 2 mg $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2$; 15 ml trace minerals [per liter: 3.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.5 g nitriloacetic acid, 1.0 g NaCl; 0.5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 0.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.1 g CaCl_2 ; 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.01 g $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$; 0.01 g H_3BO_3 and 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$]; 1 mg resazurin and 5 g starch at a pH of 6.5 (adjusted with H_2SO_4). For growth on solid medium, 0.8% agar (Difco) was added to the above medium.

The cells are grown in a fermentation vessel maintained at 75-80°C under anaerobic conditions (i.e., under nitrogen). The cells are grown for approximately 28 hours (early stationary phase). The cells are collected by centrifugation at 10,000 x g. The cell pellet may be frozen at -70°C until used. All of the subsequent operations should be carried out at 0 to 4°C unless otherwise stated.

Resuspend about 50 g of frozen *Thermotoga neapolitana* cells in 100 ml of TEDGT [50 mM Tris-HCl (pH 7.3 at 25°C), 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Tween 20] containing 2.5 mM PMSF (from 144 mM stock in DMF). The thawed and resuspended cells can be lysed in a Aminco French Pressure Cell (American Instrument Co., Silver Spring, MD, cat. no. FA-073) at 16,000 to 24,000 psi. This operation should be done twice to ensure adequate lysis. The lysate should be diluted by adding another 100 ml TEDGT containing 2.5 mM PMSF and stirring gently.

PEI (polyethyleneimine) is added to the lysate to precipitate the DNA. The exact amount of PEI is determined empirically, but in general 0.2% PEI should be adequate to precipitate most of the DNA (greater than 90%). Approximately 10 ml of 5% PEI is added to precipitate most of the DNA in the lysate. The lysate is centrifuged at 15,000 rpm for 15 minutes in a Beckman JA18 rotor to remove the precipitated DNA. The supernatant is retrieved and solid ammonium sulfate is added to 60% saturation to precipitate the DNA polymerase and other proteins. After the salt is dissolved, the sample is centrifuged at 15,000 rpm for 1 hour in a Beckmann JA18 rotor. The supernatant is discarded and the precipitated protein is gathered and dissolved in TEDGT buffer. The resolubilized protein is then placed in a dialysis membrane having a 12,000 to 14,000 mw cutoff (Spectra) and then dialyzed against TEDGT buffer to remove the ammonium sulfate.

The dialyzed protein solution is then loaded onto a 60 ml DEAE Sepharose (Sigma, St. Louis MO) column (2.5 cm i.d. x 13 cm height). The column is washed with 300 ml 0 M NaCl in TEDGT buffer. A 300 ml salt gradient is run over the column to elute the DNA polymerase starting at 0 M NaCl and ending at 0.5 M NaCl (all in TEDGT buffer). Fractions (5.0 ml) are collected and assayed for DNA polymerase activity at 74°C using the protocol described in Example 5. Fractions containing the polymerase activity are pooled, placed in dialysis membrane (12,000 to 14,000 mw cutoff; Spectra) and dialyzed against TEDGT buffer until the salt concentration is less than or equal to 0.05 M NaCl as measured by conductivity.

The pooled polymerase fraction is then loaded onto a 15 ml Heparin Sepharose (Scientific Protein Laboratory, Waunakee, WI) column (1.7 cm i.d. x 6.5 cm height). The column is washed with 150 ml 0.05 M NaCl in TEDGT buffer. A 100 ml salt gradient is run over the column to elute the DNA polymerase starting at 0.05 M NaCl and ending at 1 M NaCl (all in TEDGT buffer). Fractions (1.5 ml) are collected and assayed for DNA polymerase activity at 74°C using the assay described in Example 5. The DNA polymerase should elute between 0.14 M and 0.29 M NaCl (in TEDGT buffer). Fractions containing the polymerase activity are pooled, placed in dialysis membrane (12,000 to 14,000 mw cutoff; Spectra) and dialyzed against TEDGT buffer

until the salt concentration is less than or equal to 0.05 M NaCl as measured by conductivity.

The pooled polymerase fraction is then loaded onto a 15 ml DNA Agarose (Pharmacia) column (1.7 cm i.d. x 6.5 cm height). The column is washed with 75 ml 0 M NaCl in TEDGT buffer. A 100 ml salt gradient is run over the column to elute the DNA polymerase starting at 0 M NaCl and ending at 0.5 M NaCl (all in TEDGT buffer). Fractions (1.5 ml) are collected and assayed for DNA polymerase activity at 74°C using the assay described in Example 5. Fractions containing the polymerase activity are pooled, placed in dialysis membrane (12,000 to 14,000 mw cutoff; Spectra) and dialyzed against TEDGT buffer until the salt concentration is less than or equal to 0.05 M NaCl as measured by conductivity.

The pooled polymerase fraction is then loaded onto a 7 ml Cibracron Blue 3GA (Sigma, St. Louis, MO) column (1.25 cm i.d. x 6 cm height). The column is washed with 70 ml of 0.05 M NaCl in TEDGT buffer. A 100 ml salt gradient is run over the column to elute the DNA polymerase starting at 0.05 M NaCl and ending at 1 M NaCl (all in TEDGT buffer). Fractions (1.5 ml) are collected and assayed for DNA polymerase activity at 74°C using the assay described in Example 5. The *Tne* DNA polymerase should elute between 0.20 M and 0.46 M NaCl (in TEDGT buffer). Fractions containing the polymerase activity are pooled, placed in dialysis membrane (12,000 to 14,000 mw cutoff; Spectra) and dialyzed against storage buffer [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20].

The purity and approximate molecular weight of the DNA polymerase can be assessed by SDS-PAGE gel electrophoresis using a 4-20% gradient Tris-Glycine SDS gel (Novex, San Diego, CA). An aliquot of the purified material is mixed with sample buffer [63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% Bromphenol Blue] and the gel is run using the following running buffer [25 mM Tris-Base, 192 mM glycine, 0.1% SDS, pH 8.3]. The gel can be run for 90 minutes at 125 V D.C. until the bromphenol blue band is just to the bottom of the gel. The apparent molecular weight for *Tne* DNA polymerase should be about 97,000. Using the activity assay

described in Example 5, the number of units of DNA polymerase per microliter can be established. By visually assessing the quantity of DNA polymerase on the Coomassie stained SDS-PAGE gel compared to the protein standards run in the molecular weight marker lane, the specific activity of the DNA polymerase preparation may be estimated. The specific activity of the purified *Tne* DNA polymerase should be approximately 100,000 units/mg.

EXAMPLE 7

Characterization Of The Full-Length And Modified *Tne* Polymerases

The full-length and modified *Tne* polymerases were assayed for 5' to 3' exonuclease activity and 3' to 5' exonuclease activity.

a) 5' To 3' Exonuclease Assay

A 5' to 3' exonuclease assay was performed on the *Tne* M284 DNA polymerase (SEQ ID NO:8) present in crude lysates of cells containing the pJM284 construct (prepared as described in Example 3c) to determine whether any residual 5' to 3' exonuclease activity remained in this molecule. A comparison was made of the amount of 5' to 3' exonuclease activity present in the following thermostable DNA polymerases: *Tne* M284 (SEQ ID NO:8), the full-length r*Tne* polymerase (SEQ ID NO:2), nTaq (Promega) and UITma (Perkin Elmer). The 5' to 3' exonuclease assay was performed as follows.

End-labeled substrate DNAs were prepared by digestion of pBR322 DNA with either *Eco*RI or *Eco*RV followed by incubation with γ -³²P-ATP and T4 polynucleotide kinase. The 5' to 3' exonuclease assay was performed in a final reaction volume of 25 μ l and contained 1X Taq buffer (10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 200 μ M of each of the four dNTPs, 50 ng of labeled substrate DNA and 5 units of the DNA polymerase to be tested (a no enzyme control was also conducted; water was used in place of the enzyme). The reaction was incubated for 1 hour at 74°C. The reaction was terminated by the addition of 5 μ l of

0.5 M EDTA. Ten microliters of this mixture were spotted onto 2.3 cm circular DE81 filters (Whatman). The filters were dried briefly under a heat lamp. The filters were washed in 50 ml of 0.5 M sodium phosphate (pH 6.8) twice for 5 minutes/wash to remove unincorporated counts. The no enzyme control was used to permit
5 determination of the total cpm in the sample. The washed filters were dried under a heat lamp and then the incorporated cpm and total cpm (no enzyme control filter) were determined by liquid scintillation counting.

The results of this assay showed that nTaq and the full-length Tne (SEQ ID NO:2) polymerases contained considerable 5' to 3' exonuclease activity, while the
10 UITma and Tne M284 (SEQ ID NO:8) polymerases did not contain detectable levels of 5' to 3' exonuclease activity.

b) 3' To 5' Exonuclease Assay

3' to 5' exonuclease assays were performed in order to establish how mutations in the putative exonuclease domain of the *Tne* polymerase gene effected this
15 activity. The assay was initially performed on crude lysates from *E. coli* cells containing a plasmid which produces the Tne M284 polymerase (SEQ ID NO:8). The assays were subsequently performed on purified preparations of Tne M284 polymerase (SEQ ID NO:8).

The assay was performed using either a single stranded or double stranded
20 DNA substrate which contained a 3' end label. The substrate DNA were prepared as follows.

To create the double stranded substrate, Lambda DNA/EcoRI Markers (Promega G1721) were 3' end labeled with [α -³²P]dATP in a final reaction volume of 50 μ l containing 10 μ g of lambda DNA, 1X Buffer A [6 mM Tris-HCl (pH 7.5 at
25 37°C), 6 mM MgCl₂, 6 mM NaCl and 1 mM DTT], 5 μ l [α -³²P]dATP (3000 Ci/mmol; Amersham), 5 units Klenow fragment (Promega) and 10 mM of each of the four dNTPs. The reaction was incubated for 20 minutes at 37°C. The Klenow enzyme was inactivated by heating the mixture at 65°C for 15 min. Unincorporated counts were removed by chromatography of the reaction mixture on a Nick™ Column

(Pharmacia) according to the manufacturer's instructions. The labeled DNA was eluted in a volume of 400 μ l. A 10 μ l aliquot of the eluted DNA was counted by liquid scintillation counting and the aliquot contained approximately 2×10^5 cpm.

To create the single stranded substrate, a synthetic 74 nucleotide oligonucleotide, PM3074 (SEQ ID NO:41) is 3' end labeled with [α - 32 P]dATP in a final reaction volume of 10 μ l containing 10 pmoles of the PM3074 oligonucleotide, 1X TdT Buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM spermidine], 3 μ l [α - 32 P]dATP (3000 Ci/mmol; Amersham) and 15 units terminal deoxynucleotidyl transferase (TdT) (Promega). The reaction was incubated for 60 minutes at 37°C. The TdT enzyme was inactivated by heating the mixture at 65°C for 15 min. Unincorporated counts were removed by chromatography of the reaction mixture on a NickTM Column (Pharmacia) according to the manufacturer's instructions. The labeled DNA was eluted in a volume of 400 μ l. A 10 μ l aliquot of the eluted DNA was counted by liquid scintillation counting and the aliquot contained approximately 1×10^5 cpm.

The 3' to 5' exonuclease assay was performed as follows. In a final volume of 50 μ l, the following components were assembled, 1 or 2 units of the DNA polymerase to be assayed, 5 μ l of 10 X Toga buffer [10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20] and nuclease-free water (Promega). One tube was also set up which did not contain any polymerase (*i.e.*, a no enzyme control). The reaction mixtures were prewarmed to the reaction temperature (reactions were performed at either 25°C or 74°C) and 10 μ l of either the labeled single stranded or double stranded substrates were added to start the assay. Ten microliter fractions were removed at the following time points: 2, 4 or 6 minutes. The aliquots were spotted onto 2.3 cm circular DE81 filters and processed as described in section a) above.

The definition of one unit of 3' to 5' exonuclease activity is defined as the amount of enzyme required to remove 1 pmol of labeled 3' end from the substrate in 30 minutes.

In addition to assaying the polymerases for 3' exonuclease activity, all samples were also analyzed for DNA polymerase activity using the assay described in Example

5(b). In this manner, the ratio of 3' exonuclease activity to polymerase activity could be determined for each polymerase.

When the 3' to 5' exonuclease assay was performed using on crude lysates containing the Tne M284 enzyme, the assay was run using only the single stranded substrate at 25°C. Under these conditions, a comparison was made between purified *UITma* (a modified form of *Tma* polymerase which lacks 5' to 3' exonuclease activity) and *UITma* spiked into a lysate derived from *E. coli* cells shown to lack 3' to 5' exonuclease activity; the spiked sample showed a 22% lower 3' exonuclease: polymerase activity ratio (polymerase activity was measured using the assay described in Example). As *E. coli* lysate alone shows no 3' exonuclease activity, this result indicated that results obtained with crude lysates containing the Tne M284 protein (SEQ ID NO:8) should underestimate the 3' to 5' exonuclease levels that would be seen using purified *Tne* polymerase preparations.

When purified *UITma*TM was compared with crude lysates containing the full-length (SEQ ID NO:2) or Tne M284 (SEQ ID NO:8) polymerases, the highest exonuclease:polymerase activity ratio was seen for the full-length *Tne* enzyme (1.37), then M284 Tne (0.83), then *UITma*TM (0.45). These results were unexpected as it was not predictable that a deletion in the 5' to 3' exonuclease domain (Tne M284 polymerase) would result in a 39% decrease in 3' to 5' exonuclease activity.

Purified Tne M284 polymerase was used in 3' exonuclease assays in comparison to purified *UITma*TM using both single stranded and double stranded substrates at 25°C and 74°C. Surprisingly, both enzymes displayed the same level of 3' exonuclease activity on single stranded substrate at 25°C. Results obtained using crude lysates containing Tne M284 polymerase suggested that Tne M284 would have a higher activity. Both enzymes (purified Tne M284 and *UITma*TM) gave nearly identical results when the 3' exonuclease assay was performed using the double stranded substrate at 25°C (and both showed very low activity under these conditions).

When the 3' exonuclease assay was performed using a single stranded substrate at 74°C, *UITma*TM had no activity, whereas purified Tne M284 showed an exonuclease: polymerase ratio of 0.32.

These results demonstrate that purified Tne M284 polymerase and *UITma*TM have significantly different 3' to 5' exonuclease activities.

EXAMPLE 8

Characterization Of The Purified *Tne* Polymerases

In order to ascertain the characteristics of the full length and modified forms of the *rTne* polymerases, a number of determinations were made as described below.

a) Molecular Weight On SDS-PAGE Gels

The apparent molecular weight of the full length and modified *rTne* polymerases were determined by SDS-PAGE. An aliquot (10 units) of each purified polymerase was mixed with sample buffer [63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue] and applied to a precast 4-20% gradient Tris-Glycine SDS gel (Novex, San Diego, CA). Molecular weight markers (Promega) were run on the same gel to allow calculation of the molecular weight of the *Tne* polymerases. The gel was run using the following running buffer [25 mM Tris-Base, 192 mM glycine, 0.1% SDS, pH 8.3] for 90 minutes at 125 V D.C. until the bromphenol blue band was just to the bottom of the gel.

Following electrophoresis, the gel was stained with Coomassie blue to visualize the proteins. The full-length *rTne* polymerase migrated with an apparent molecular weight of 97,000 daltons. Tne M284, Tne M284(D323E), Tne M284(E325D), Tne M284(Y464F), Tne M284(D468N), and Tne M284(D323A, D389A) all migrated with an apparent molecular weight of 70,000 daltons.

b) Specific Activity Of Purified *Tne* Polymerases

Preparations of purified full-length *rTne* and *Tne* M284, *Tne* M284(D323E), *Tne* M284(E325D), *Tne* M284(Y464F), *Tne* M284(D468N), and *Tne* M284(D323A, D389A) were all found to have a specific activity of 100,000 units/mg when the enzymes were purified and DNA polymerase assays were conducted as described in Example 5.

c) 3' To 5' Exonuclease Activity

Purified preparations of full-length *rTne* and *Tne* M284, *Tne* M284(D323E), *Tne* M284(E325D), *Tne* M284(Y464F), *Tne* M284(D468N) and *Tne* M284(D323A, D389A) were assayed for 3' to 5' exonuclease activity. In the same experiment, the 3' to 5' exonuclease activity of the *UITma*TM (Perkin Elmer) and *nTaq* (Promega) polymerases were also measured for comparison to the *Tne* polymerases.

The 3' exonuclease assay involved the incubation of the above enzymes with a 3' end-labeled DNA substrate; the loss of radioactivity from the labeled substrate was measured after a 10 minute incubation period.

i) Preparation Of The 3' End-Labeled Substrate

Ten micrograms of lambda phage DNA was digested with 50 u of *Mlu*I (Promega) in a 50 μ l reaction volume in 1X Buffer D [6 mM Tris-HCL (pH 7.9 at 37°C), 6 mM MgCl₂, 150 mM NaCl and 1 mM DTT]. The reaction was incubated for 3 hours at 37°C. The 3' ends were then filled in using [α -³²P]-dCTP and unlabelled dGTP in a reaction containing 5 u of Klenow Exo- (USB) and 1X Buffer D in a volume of 100 μ l; the reaction mixture was incubated for 20 min at 25°C. The reaction was terminated by heating the sample to 74C for 15 min. The bulk of the unincorporated [α -³²P]-dCTP was removed by passage of the sample over a NickTM column (Pharmacia) and the labeled DNA was eluted in 400 μ l TE [10 mM Tris-HCl

(pH 7.4), 1 mM EDTA]. An aliquot (10 μ l) of the eluted DNA was counted in a scintillation counter and the aliquot contained approximately 2×10^5 cpm.

ii) 3' To 5' Exonuclease Assay

The 3' to 5' exonuclease assay was performed as follows. In a final volume of 50 μ l, the following components were assembled, 1 or 2 units of the DNA polymerase to be assayed, 5 μ l of 10 X Toga buffer [10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20] and nuclease-free water (Promega). One tube was also set up which did not contain any polymerase (*i.e.*, a no enzyme control). The reaction mixtures were prewarmed to the reaction temperature (reactions were performed at either 25°C or 74°C) and 10 μ l of the labeled double stranded substrates were added to start the assay. Ten microliter fractions were removed at the following time points: 2, 4, 6 and 10 minutes (In a subsequent experiment, aliquots were withdrawn at 30 and 60 minutes to permit the detection of activity for enzymes showing very low levels of activity). The aliquots were spotted onto 2.3 cm circular DE81 filters and processed as described in Example 7, section a) above.

The definition of one unit of 3' to 5' exonuclease activity is defined as the amount of enzyme required to remove 1 pmol of labeled 3' end from the substrate in 30 minutes.

In addition to assaying the polymerases for 3' exonuclease activity, all samples were also analyzed for DNA polymerase activity using the assay described in Example 5(b). In this manner, the ratio of 3' exonuclease activity to polymerase activity could be determined for each polymerase. The results are reported as the ratio of 3' exonuclease activity to polymerase activity. The value obtained for the full-length Tne polymerase was assigned a value of 100% and all other values are expressed relative to this value.

TABLE 3

3' Exonuclease Activity

DNA Polymerase	Exo:Pol Ratio
<i>rTne</i>	100 ± 9.0
<i>Ultma</i> TM	23.3 ± 0.8
Tne M284	28.0 ± 0.08
Tne M284(Y464F)	1.9 ± 0.2
Tne M284(D468N)	0.47 ± 0.02
Tne M284(D323E)	0.0
Tne M284(E325D)	0.0
Tne M284(D323A, D389A)	0.0
<i>nTaq</i>	0.0

The results shown in Table 3 demonstrate that deletions which remove 5' exonuclease activity (e.g., Tne M284) also affect 3' exonuclease activity in the *Tne* polymerases (compare *rTne* with Tne M284; activity of Tne M284 is roughly one third that seen in *rTne*). Mutation of amino acid residues suspected of being critical for 3' exonuclease activity (by analogy to similar residues in *E. coli* polymerase I) further reduces or eliminates 3' exonuclease. The virtual elimination of 3' exonuclease activity seen with the Tne M284(D468N) polymerase was unexpected. The analogous mutation in the Klenow fragment of DNA polymerase I (D501N) reduces 3' exonuclease activity only by 2-fold [Derbyshire *et al.*, EMBO J. 10:17 (1991)]. These results underscore the fact that much remains to be learned about structure-function relationships and that one cannot predict, with certainty, the effect of a given mutation based on analogy to other proteins.

d) **Thermostability Of The Purified *Tne* Polymerases**

At 97.5°C

Thermostability was measured by incubating a DNA polymerase at 97.5°C for various amounts of time and measuring the remaining DNA polymerase activity at 74°C. Samples were withdrawn after 0, 5, 10, 30, 60, 90, and 120 minutes after exposure to 97.5°C and DNA polymerase assays were conducted as described in Example 5b. The time necessary to reduce the activity by one-half the initial value was determined by plotting the remaining activity versus the time of incubation at 97.5°C.

The incubation buffer contained 10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20 and 1.5 mM MgCl₂ and 2-3 units of the enzyme to be tested. All enzymes, including commercially available enzymes (*e.g.*, nTaq, *UITma*TM), were assayed using the DNA polymerase assay described in Example 5. The results of the thermostability assays are summarized in Table 4 below.

TABLE 4

Thermostability of *Tne* Polymerases

DNA polymerase	SEQ ID NO:	Half-life at 97.5°C (min)
full-length rTne	2	5
Tne M284 (E325D)	19	5
native Taq		8
<i>UITma</i> TM		12
Tne M284(D323E)	16	12.5
Tne M284(Y464F)	23	16
Tne M284	8	18
Tne M284(D323A, D389A)	35	22
Tne M284 (D468N)	26	66

The results shown in Table 4 demonstrate that deletion of the amino terminal 5' to 3' exonuclease domain of *Tne* polymerase increases resistance to thermal degradation (2.5 to 13.2 fold). While amino-terminal deletions have been shown to increase thermostability (*i.e.*, thermal tolerance) of certain modified forms of *Taq* DNA polymerase (*e.g.*, the Stoffel fragment) and *Tma* DNA polymerase, the increase seen is generally about 2-3 fold. The increase in thermostability seen by the introduction of single or double point mutations into the *Tne* M284 deletion mutant was unexpected, especially the dramatic increase in thermostability caused by the single point mutation present in *Tne* M284(D468N) (SEQ ID NO:26).

The Tne M284(D468N) protein is identical to the Tne M284 protein with the exception that an asparagine is substituted for an aspartate at position 468; the carbon backbones of these two molecules should be identical, yet there is a greater than 5-fold resistance to thermal degradation seen when asparagine is present at position 468. The only difference between these two molecules should be a negatively charged carbonyl group (aspartate) and a neutral amide group (asparagine); both of these groups can participate in hydrogen-bonding. Substitutions of solvent-exposed amino acids (*e.g.*, aspartate or asparagine) have been shown to have little effect on protein stability or structure [Matthews, *Ann. Rev. Biochem.* 62:139 (1993)], leading to the view that the rigid parts of proteins are critical for folding and stability. Based on analogy to the *E. coli* DNA polymerase I molecule at position 501, it is expected that position 468 in the *Tne* DNA polymerase is accessible to solvent. Furthermore, since Tne M284(D468N) lacks the carbonyl group to coordinate a metal ion, it was predicted that a slight destabilization (in response to heat) would be seen rather than a dramatic stabilization. This data show that a subtle change in the 3' exonuclease active site can dramatically alter the thermostability (*i.e.*, thermotolerance) of the *Tne* DNA polymerase.

The above results demonstrate that several of the modified *The* polymerases have superior thermostability; accordingly, when these enzymes are used in PCR and other reactions run at elevated temperature, less polymerase activity needs to be used as less enzyme is inactivated by exposure to elevated temperature.

g) **Optimal Temperature For DNA Polymerase Activity**

The DNA polymerase activity of several *Tne* polymerases and nTaq DNA polymerase was measured at various temperatures using the assay described in Example 5b (with the exception that the temperature of incubation was varied). The results are summarized in Table 5. The temperature which gave the highest activity for a given enzyme was assigned a value of 100% and all other values given are expressed relative to the 100% value.

The results shown in Table 5 demonstrate that for nTaq DNA polymerase, the maximal DNA polymerase activity was present when the reaction was run at 78°C. Optimal temperature for DNA polymerase activity for the full-length rTne DNA polymerase was 74°C. Optimal temperature for DNA polymerase activity for the *Tne* M284 and *Tne* M284(D323A, D389A) DNA polymerases was 75°C.

TABLE 5

Optimal Temperature For *Tne* Polymerase Activity

Temp. (°C)	<i>Tne</i> M284	<i>Tne</i> M284(D323A, D389A)	nTaq	rTne
40	12	15	n.d.	n.d.
45	15	17	n.d.	n.d.
50	18	20	n.d.	n.d.
55	24	26	n.d.	n.d.
60	33	36	n.d.	n.d.
65	53	52	n.d.	n.d.
70	71	80	n.d.	77
72	n.d.	n.d.	n.d.	86
74	n.d.	n.d.	n.d.	100
75	100	100	n.d.	n.d.

TABLE 5

Optimal Temperature For *Tne* Polymerase Activity

Temp. (°C)	Tne M284	Tne M284(D323A, D389A)	nTaq	rTne
76	n.d.	n.d.	93	94
77	n.d.	n.d.	97	n.d.
78	n.d.	n.d.	100	96
79	n.d.	n.d.	97	n.d.
80	53	56	85	85
81	n.d.	n.d.	82	n.d.
82	n.d.	n.d.	68	68
85	16	16	n.d.	n.d.
90	8	8	n.d.	n.d.

EXAMPLE 9

The *Tne* DNA Polymerases Provide Improved
Enzymes For Use In The Polymerase Chain Reaction

The use of the full-length and modified forms of *Tne* polymerase in the PCR was examined. The results of the following experiments show that the *Tne* polymerases provide improved enzymes for a variety of PCR applications.

**a) The Modified Tne Polymerases Utilize A Broader
Range Of Optimal dNTP Concentrations In The PCR**

PCR reactions were conducted using a range of dNTP concentrations to determine the optimal range of dNTP concentrations utilized by either the *UITma*TM DNA polymerase (Perkin Elmer) or the Tne M284 and Tne M284(D323A, D389A)

polymerases. The modified *Tne* polymerases were found to produce high yields of amplified product over a much broader range of nucleotide concentrations. The PCRs were conducted as follows.

The three enzyme preparations were assayed for DNA polymerase activity using the assay in Example 5b so that the same amount of enzyme was used in the PCRs. PCRs were performed in a buffer optimized for each type of enzyme; the *UITma*TM and *Tne* enzymes were both found to give optimal results in the following buffer [10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20]. All reactions contained 1 ng of pGEM-luc (Promega) as the template, 20 pmol of each primer [the primers used were LME41(SEQ ID NO:42) and LME43(SEQ ID NO:43)], 1.5 mM MgCl₂ (this concentration was chosen as it was optimal for both the *UITma*TM and the modified *Tne* enzymes as shown below in section b) and 2.5 units of each enzyme. The final reaction volume was 50 µl.

A dilution series was created for the mixture of all four dNTPs ranging from 20 to 200 µM (20, 40, 60, 80, 100, 120, 140, 160 and 200 µM). The PCRs were thermal cycled using the following conditions, an initial denaturation at 96°C for 2 min, followed by 30 cycles comprising denaturation at 94°C for 30 sec, annealing/extension at 65°C for 2 min; following the last cycle the tubes were incubated at 65°C for 10 min and then the tubes were incubated at 4 °C. Thermal cycling was conducted on a Perkin Elmer Thermocycler Model 480. The PCR products were resolved on 1% agarose gels, stained with EtBr and quantitated by visual inspection of the stained gels. The experiment was repeated a second time and similar results were obtained.

The optimal dNTP concentration for *UITma*TM was found to be 40 µM (the same value as reported to be optimal by Perkin Elmer in the *UITma*TM product insert);

slight levels of product could be detected using the *UITma*TM enzyme at a concentration of 20 μ M dNTPs but not at all at other concentrations tested. The optimal range of concentrations of dNTPs for Tne M284(D323A, D389A) was found to be from 60 μ M to 200 μ M with slight amounts of product being detected at both 20 and 40 μ M dNTP. The optimal range of concentrations of dNTPS for Tne M284 was from 40 μ M to 200 μ M with slight amounts of product being detected at 20 μ M dNTP.

The ability to use a wide range of dNTP concentrations is advantageous. The modified *Tne* polymerases allow PCR users wide flexibility in the design of the reaction conditions. Because the modified *Tne* polymerases are not sensitive to small variations in dNTP concentration, PCRs using these enzymes are more robust. Additionally the ability to use a higher concentration of dNTPs may also allow a higher yield of product to be generated in a PCR using the modified *Tne* polymerases as compared to the use of the *UITma*TM polymerase.

b) The *Tne* Polymerases Tolerate A Broader Range Of Mg⁺⁺ Concentrations In PCR

In side-by-side tests with *UITma*TM DNA polymerase and Tne M284 or Tne M284(D323A, D389A), the *Tne* variants were found to produce high yields of amplified product over a broader range of magnesium ion concentrations. A dilution series was created for magnesium (MgCl₂) ranging from 0.5 to 5 mM (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 mM). The other components of the PCR were as described above in section a) with the exception that a single dNTP concentration was used (40 μ M dNTP was used for the *UITma*TM enzyme and the modified *Tne* enzymes as this value was within the operable range for all three enzymes; it is noted that 40 μ M

dNTP is not optimal for the Tne M284(D323A, D389A enzyme and that even higher product yields would be expected if 60 μ M dNTP were used for this enzyme) and the concentration of $MgCl_2$ was varied. Thermal cycling conditions were as described above in section a). The reaction products were treated as described above in section a).

The results of these assays showed that the *Ultma*TM enzyme (Perkin Elmer) had an optimal concentration of 1.5 mM $MgCl_2$ and a great deal of non-specific background products were seen on the gels (as a smear). The optimal concentration for Tne M284 enzyme was found to be from 1 to 3 mM $MgCl_2$ and some nonspecific background products were observed. The optimal concentration for M284(D323A, D389A) was from 1 to 3 mM $MgCl_2$ and very little or no detectable non-specific background products were observed.

The ability of a thermostable enzyme to produce only specific PCR products over a wide range of Mg^{++} concentrations is important for certain PCR applications. For example, multiplexing PCRs utilize several pairs of primers to amplify several different targets in the sample. As each primer pair and target combination will have a optimum Mg^{++} concentration and this value may vary considerably from primer set to primer set, the availability of thermostable polymerases having a wide optimum for Mg^{++} is advantageous. Therefore, the Tne M284 and Tne M284(D323A, D389A) polymerases provide improved thermostable DNA polymerases.

c) **Modified *Tne* Polymerases Produce High PCR**

Product Yields

A comparison was made between the yield of PCR product obtained using a consistent amount of polymerase activity when the following enzymes were used in the PCR: *Tne* M284, *Tne* M284(D323E), *Tne* M284(E325D), *Tne* M284(Y464F), *Tne* M284(D468N), *Tne* M284(D323A, D389A), AmpliTaq (Perkin Elmer), nTaq (Promega) and *Ultma*TM (Perkin Elmer). All enzymes were assayed using the DNA polymerase assay described in Example 5b so that the same number of units of enzyme were added to the PCRs.

PCRs which used *Ultma*TM polymerase contained 10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20 and 1.5 mM MgCl₂. This buffer gives optimal PCR results for the *Ultma*TM enzyme (as reported by Perkin Elmer). For the results shown in Table 6, PCRs which used the *Tne* DNA polymerases contained 10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20 and 1.5 mM MgCl₂. This buffer gives acceptable results for the *Tne* polymerases.

For the results shown in Table 7, PCRs which used the *Tne* DNA polymerases contained 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 0.01% Tween 20 and 1.5 mM MgCl₂ as it was found that the *Tne* DNA polymerases perform better when the pH of the buffer is raised to 9.0 and the amount of detergent (*i.e.*, Tween 20) is increased to 0.01%.

PCRs which used the *Ultma*TM enzyme contained 40 μ M dNTPs as this value was determined to be optimal for this enzyme (see section a, above). PCRs which used the modified *Tne* enzymes contained 140 μ M dNTPs as this value was determined to be optimal for these enzymes (see section a, above).

PCRs which used nTaq or AmpliTaq polymerase contained 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100; this buffer gives optimal PCR results for the nTaq and AmpliTaq polymerases. PCRs which used the nTaq and AmpliTaq enzymes contained 200 μM dNTPs as this value is reported to be optimal for these enzymes.

A single template, pGEM-luc was tested in conjunction with two different primer pairs. The primer pair comprising LME41 (SEQ ID NO:42) and LME45 (SEQ ID NO:44) amplifies a 500 bp target on the template. The primer pair comprising LME41 (SEQ ID NO:42) and LME43 (SEQ ID NO:43) amplifies a 1.5 kb target on the template.

All PCR reactions contained 3.0 units of the enzyme to be tested, 2 ng of pGEM-luc (Promega) as the template, 50 pmol of each primer pair, 1.5 mM MgCl₂ (this concentration was chosen as it was determined to be optimal for all three types of enzyme tested using this template and these primer pairs). The final reaction volume was 100 μl.

The PCRs which amplified the 500 bp target were thermal cycled using the following conditions, an initial denaturation at 96°C for 2 min, followed by 25 cycles comprising denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 1 min; following the last cycle the tubes were incubated at 4°C.

The PCRs which amplified the 1500 bp target were thermal cycled using the following conditions, an initial denaturation at 94°C for 2 min, followed by 30 cycles comprising denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min; following the last cycle, the tubes were incubated at 68°C for 10 min and then the tubes were brought to 4°C.

Thermal cycling was conducted on a Perkin Elmer Thermocycler Model 480. Each reaction was run in duplicate. The PCR products were resolved by electrophoresis through 1% agarose gels, followed by staining with EtBr and quantitated by scanning the gels with a fluoroimager (Molecular Dynamics); product yields were quantitated using ImageQuant software (Molecular Dynamics). The results (average of the duplicates) are shown below in Tables 6 and 7. The enzyme which gave the highest yield was assigned a value of 100% and all other values are expressed as a percentage of the maximal yield.

The results shown above in Tables 6 and 7 demonstrate the following. Regardless of the target size, the modified *Tne* enzymes produced higher yields as compared to the *Ultma*TM enzyme (2.5 to 6.7 fold more product). The modified *Tne* enzymes also performed better than nTaq when the 500 bp target was used (about 2 fold more product) and better than the recombinant *Taq* polymerase (AmpliTaq) when the 1.5 kb target was used (about 30% more product).

Thus, to produce the same amount of PCR product, fewer units of modified *Tne* polymerases are required compared to the use of the *Ultma*TM, nTaq and AmpliTaq enzymes. These modified *Tne* polymerases allow the user to use smaller amounts of enzyme in the PCR and therefore will provide significant cost savings in PCR applications.

TABLE 6

Product Yields For Amplification Of A 500 bp Target

Enzyme	% Yield
nTaq	59
<i>Ultma</i> [™]	16
Tne M284	100
Tne M284(D323E)	89
Tne M284(E325D)	87
Tne M284(Y464F)	91
Tne M284(D468N)	89
Tne M284(D323A, D389A)	91

TABLE 7

Product Yields For Amplification Of A 1.5 kb Target

Enzyme	% Yield
AmpliTaq	67
<i>Ultma</i> [™]	40
Tne M284	81
Tne M284 (D323E)	70
Tne M284 (E325D)	86
Tne M284 (Y464F)	93
Tne M284 (D468N)	100
Tne M284 (D323A, D389A)	87

EXAMPLE 10

Thermal Cycle Sequencing Using Modified *Tne* Polymerases

5 The modified *Tne* polymerases were further characterized by examining their ability to be used in thermal cycle sequencing reactions. Initial sequencing reactions were performed using with the *Tne* M284(D323E) and *Tne* M284(E325D) mutants (SEQ ID NOS:16 and 19, respectively) using the buffer and dideoxy mixes from the fmol® DNA Sequencing System kit (Promega) according to the manufacturer's instructions. The buffer and concentrations of dideoxy and deoxyribonucleotides in this kit were optimized for use with sTaq DNA polymerase (Promega; sTaq is a modified form of *Taq* DNA polymerase which has very low 5' to 3' exonuclease activity).

10 When the sequencing reactions were performed using purified preparations of either *Tne* M284(D323E) (SEQ ID NO:16) or *Tne* M284(E325D) (SEQ ID NO:19), the resulting reaction products were very short and uneven and created light bands on the sequencing gel in every position and in every lane. The light bands in every position were consistent with the presence of residual 3' to 5' exonuclease activity in the modified *Tne* polymerases.

15 Thermal cycle sequencing reactions were then performed using the triple mutant *Tne* M284(D323A, D389A) (SEQ ID NO:35). All thermal cycle sequencing was carried out using a Perkin-Elmer 9600 thermal cycler in conjunction with the fmol® DNA Sequencing System reagents (Promega). The template used in the sequencing reactions was the *Tne* clone pE325D (Example 4b) and the 5' labeled primer was the JH66 primer (SEQ ID NO:12). The reactions were run at 95°C for 15 seconds and 70°C for 60 seconds for 30 cycles.

20 The results of this experiment showed that the use of the triple mutant enzyme (SEQ ID NO:35) eliminated the light bands in every position which were seen with *Tne* M284(D323E) and *Tne* M284(E325D); however the sequence ladders were still shifted toward short extension products. These short intense reads were indicative of a

DNA polymerase having a higher affinity for the dideoxynucleotides than *Taq* DNA polymerase.

To examine if the triple mutant *Tne* enzyme (SEQ ID NO:35) had a higher affinity for dideoxynucleotides, the sequencing reactions were repeated using a lower ratio of dNTPs to ddNTPs in the sequencing reaction mixtures (all other conditions remained the same). Table 8 lists the 3X mixes used for sTaq and *Tne* M284(D323A, D389A) polymerases in this experiment. The use of lower concentration of dideoxynucleotides in the sequencing reaction run using the triple mutant *Tne* enzyme (SEQ ID NO:35) produced reads as long as those obtained using sTaq.

TABLE 8

	sTaq		<i>Tne</i> M284, D323A, D389A	
	dNTPs	ddNTPs	dNTPs	ddNTPs
G mix	20 μ M	30 μ M	20 μ M	30 μ M
A mix	20 μ M	350 μ M	20 μ M	75 μ M
T mix	20 μ M	600 μ M	40 μ M	40 μ M
C mix	20 μ M	200 μ M	40 μ M	20 μ M

EXAMPLE 11

Optimization Of Nucleotide Mixtures For Thermal Cycle Sequencing Using *Tne* M284(D323A, D389A) Polymerase

The results discussed in Example 10 demonstrated that the *Tne* M284(D323A, D389A) polymerase (SEQ ID NO:35) has a higher affinity for dideoxynucleotides than does sTaq polymerase. Further testing with various concentrations of dideoxy- and deoxynucleotides, including modified dNTPs, in sequencing reactions was performed to ascertain the optimal concentration for these reagents. The optimized *Tne* dNTP/ddNTP mixes are detailed in Table 9 below. The values reported in Table 9 represent 3X mixtures; these mixtures are diluted 3-fold in the final reaction mixture

as described below. To obtain the final concentration of dNTPs and ddNTPs in the reactions, the values in Table 9 are divided by 3.

TABLE 9

Optimized Nucleotide Mix Formulation for *Tne* Polymerase

Component	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	20 μ M	-	-	-
ddATP	-	50 μ M	-	-
ddTTP	-	-	75 μ M	-
ddCTP	-	-	-	25 μ M
7-deaza dGTP	30 μ M	30 μ M	30 μ M	30 μ M
dATP	30 μ M	30 μ M	30 μ M	30 μ M
dTTP	30 μ M	30 μ M	30 μ M	30 μ M
dCTP	30 μ M	30 μ M	30 μ M	30 μ M

To illustrate the fact that the *Tne* M284(D323A,D389A) enzyme has a higher affinity for ddNTPs as compared to sTaq, the following thermal cycle sequencing reactions were performed. The template used was pGEM-3Zf(+) (Promega) and the primer was the γ -³²P end labeled pUC/M13 Forward Primer (Promega; SEQ ID NO:40). Reactions using sTaq polymerase or the *Tne* M284(D323A, D389A) polymerase were performed using dNTP/ddNTP mixes optimized for sTaq as provided in the fmol® DNA Sequencing System.

The extension products of thermal cycle sequencing reactions performed using either sTaq or the *Tne* M284(D323A, D389A) polymerase were resolved by electrophoresis on a 6% denaturing polyacrylamide gel; following electrophoresis, the gel was exposed to X-ray film. The resulting autoradiograph is shown in Figure 5, Panel A.

In Figure 5, Panel A, lanes 1-4 contain reaction products generated using sTaq and lanes 5-8 contain reaction products generated using the Tne M284(D323A, D389A) polymerase. In each set of four lanes, reactions run in the presence of ddGTP, ddATP, ddTTP and ddCTP were loaded left to right.

5 The results shown in Figure 5, Panel A show that the sequence ladders generated using the modified Tne polymerase were shifted toward short extension products. These short intense reads were indicative of a DNA polymerase having a higher affinity for the dideoxynucleotides than *Taq* DNA polymerase.

10 Sequencing reactions were then performed using sTaq in conjunction with dNTP/ddNTP mixes optimized for sTaq as provided in the fmol® DNA Sequencing System or the Tne M284(D323A, D389A) polymerase and the dNTP/ddNTP mixes shown in Table 9. The template used was pGEM-3Zf(+) (Promega) and the primer was the γ -³²P end labeled pUC/M13 Forward Primer (Promega; SEQ ID NO:40). An autoradiograph of reaction products is shown in Figure 5, Panel B.

15 In Figure 5, Panel B, lanes 1-4 contain reaction products generated using sTaq and sequencing mixes optimized for sTaq and lanes 5-8 contain reaction products generated using the Tne M284(D323A, D389A) polymerase and sequencing mixes shown in Table 9 for the Tne M284(D323A, D389A) polymerase. In each set of four lanes, reactions run in the presence of ddGTP, ddATP, ddTTP and ddCTP were loaded
20 left to right.

The results shown in Figure 5, Panel B demonstrate that the Tne M284(D323A, D389A) polymerase has a higher affinity for ddNTPs than does sTaq polymerase and therefore lower concentrations of ddNTPs must be used in the sequencing reactions.

25 Using the optimized mixes shown in Table 9, the Tne M284(D323A,D389A) enzyme was compared with Sequencing Grade Taq (sTaq; Promega) for its ability to sequence three different templates. sTaq (Promega) was used in conjunction with the fmol DNA Sequencing System (Promega) and all protocols were followed as per the instructions. The Tne M284(D323A,D389A) polymerase was used in conjunction with the fmol® DNA Sequencing System kit (Promega), except that the dNTP's/ddNTP's
30 mixes used were the Tne optimized mixes shown in Table 9.

The following three DNA templates used in the indicated amounts in the thermal cycle sequencing reactions. Forty femtomoles of pGEM-3Zf(+) (Promega) was sequenced using the γ -³²P end labeled pUC/M13 Forward Primer (Promega; SEQ ID NO:40). Four femtomoles of a 500 bp PCR fragment was sequenced using the gamma 32P end labeled LME-28 primer (5'- TAATACGACTCACTATAGGGC GAAT-3' (SEQ ID NO:47). Four femtomoles of λ gt11 phage DNA (Promega) was sequenced using a γ -³²P end labeled λ gt11 Forward Primer (Promega).

The 500 bp PCR product used as template was generated by amplification of the template pGEM-luc with primers LME41 (SEQ ID NO:42) and LME45 (SEQ ID NO:44) as described in Example 9.

The thermal profile of all sequencing reactions was 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 70°C for 60 sec; following the last cycle, the reactions were brought to 4°C. The thermal cycling was performed using a Perkin-Elmer 480 Thermal Cycler. The extension products were separated on a 6% denaturing polyacrylamide gel; following electrophoresis, the gel was exposed to X-ray film. The resulting autoradiographs are shown in Figure 6.

In Figure 6, three panels are shown. Panel A contains the extension products generated using pGEM-3Zf(+) as the template; Panel B contains the extension products generated using the 500 bp PCR product as the template; Panel C contains the extension products generated using λ gt11 phage DNA as the template. In all three panels, eight lanes are shown. The first four lanes correspond to extension products generated using sTaq polymerase; the last four lanes correspond to extension products generated using the Tne M284(D323A, D389A) polymerase. In each set of four lanes, the termination mixes were used in the following order (left to right): G, A, T and C.

The results shown in Figure 6 demonstrated that the Tne M284(D323A, D389A) polymerase produced sequencing ladders which were virtually identical in terms of quality and quantity to those produced using sTaq (Promega); both enzymes provided the correct DNA sequence for each template. While both enzymes produced similar results in thermal cycle sequencing reactions, the Tne M284(D323A, D389A) polymerase required lower concentrations of dideoxynucleotides. Thus, the use of the

use of the Tne M284(D323A, D389A) polymerase will result in considerable cost savings for thermal cycle sequencing applications.

The optimal ddNTP concentrations shown in Table 9 for the Tne M284(D323A, D389A) polymerase (SEQ ID NO:35) as compared to the optimal concentrations for sTaq (Table 8) demonstrate that the modified *Tne* polymerase has a greater affinity for all four ddNTPs. In particular this modified *Tne* polymerase requires 8-fold less ddTTP or ddCTP, 7-fold less ddATP and 1.5-fold less ddGTP than does sTaq in thermal cycle sequencing reactions. As dideoxynucleotides are an expensive component of the sequencing reaction mixtures, the use of the Tne M284(D323A, D389A) polymerase (SEQ ID NO:35) in place of enzymes such as *Taq* polymerase which have lower affinities for ddNTPS will result in considerable cost savings. These results also demonstrate that the Tne M284(D323A, D389A) enzyme can utilize the nucleotide analog 7-deaza dGTP which is used to resolve band compression artifacts generated when sequencing G+C-rich regions of DNA.

EXAMPLE 12

Preferred Sequencing Protocol Using Tne M284(D323A, D389A) Polymerase

The preferred sequencing protocol uses a thermal cycling format. A detectable signal may be generated using either an end radiolabeled primer or a radiolabeled dNTP that is incorporated into the extension products.

a) Sequencing Protocol Using An End-Labeled Primer

i) Primer Radiolabeling Reaction

To generate a radiolabeled primer for use in the sequencing reaction, the following reaction components are assembled in a 0.5 ml microcentrifuge tube: 10 pmol of the desired sequencing primer; 10 pmol of γ -labeled ATP (see Table 10 for amount); 1 μ l of 10X T4 polynucleotide kinase Buffer [500 mM Tris-HCl (pH 7.5); 100 mM $MgCl_2$; 50 mM DTT; 1.0 mM spermidine]; 5 units T4 polynucleotide kinase and sterile H_2O to a final volume of 10 μ l. The reaction mixture is incubated at 37°C

for 10-30 min (if end-labeling is to be performed using $[\gamma\text{-}^{35}\text{S}]\text{ATP}$, 20 units of polynucleotide kinase are used and the reaction is incubated for 4 hours at 37°C). The reaction is then terminated by inactivation of the kinase by incubation at 90°C for 2 minutes. The tube is then briefly spun in a microcentrifuge to collect any condensation. The labeled primer may be used directly in the sequencing reaction without further purification.

TABLE 10

Amount of Radiolabeled Nucleotide Needed To Equal 10 pmol

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$:	3.0 μl of 3,000Ci/mmol,	10 $\mu\text{Ci}/\mu\text{l}$
	5.0 μl of 5,000Ci/mmol,	10 $\mu\text{Ci}/\mu\text{l}$
	0.5 μl of 6,000Ci/mmol,	135 $\mu\text{Ci}/\mu\text{l}$
$[\gamma\text{-}^{35}\text{S}]\text{ATP}$:	1.4 μl of 1,326Ci/mmol,	10 $\mu\text{Ci}/\mu\text{l}$

ii) Extension/Termination Reactions

For each set of sequencing reactions, label four 0.5 ml microcentrifuge tubes (G, A, T, C). Add 2 μl of the appropriate 3X dNTP/ddNTP Mix to each tube (see Table 9 for components of the 3X mixes). Cap the tubes and store on ice or at 4°C until needed. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 4-40 fmol of template DNA (see Table 11 below for recommended amounts); 5 μl *fmol*® Sequencing 5X Buffer [250 μM Tris-HCl (pH 9.0 at 25°C), 10 mM MgCl_2]; 1.5 μl labeled primer (1.5pmol); sterile H_2O to a final volume of 16 μl (this comprises the primer/template mix).

Add 1.0 μl of Tne M284 (D323A,D389A) DNA Polymerase (5u/ μl) to the primer/template mix. Mix briefly by pipetting up and down (this comprises the enzyme/primer/template mix). Add 4 μl of the enzyme/primer/template mix to the inside wall of each tube containing d/ddNTP mix. Add one drop (approximately 20 μl) of mineral oil to each tube and briefly spin in a microcentrifuge. Place the reaction tubes in a thermal cycler that has been preheated to 95°C and start the cycling program.

TABLE 11

Recommended Amounts Of Template DNA (ng) for End-Labeled Primer Protocol

Template Length	ng of Template
200bp (PCR product)	0.5ng (4fmol)
3,000-5,000bp (supercoiled plasmid DNA)	100ng (40fmol)
48,000bp (lambda DNA)	130ng (4fmol)

When the primer used is less than 24 nucleotides in length or has a G+C-content less than 50%, the following cycling profile is used: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds (denaturation); 42°C for 30 seconds (annealing); 70°C for 1 minute (extension); the tubes are then brought to 4°C.

When the primer used is greater than or equal to 24 nucleotides in length or when shorter primers having a G+C-content greater than or equal to 50%, the following cycling profile is used: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds (denaturation); 70°C for 30 seconds (annealing/extension); the tubes are then brought to 4°C.

After the thermocycling program has been completed, add 3 μ l of *fmol*[®] Sequencing Stop Solution [10 mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanol] to the inside wall of each tube. Briefly spin the tubes in a microcentrifuge to terminate the reactions. Heat the reactions at 70°C for 2 minutes immediately before loading onto a sequencing gel. Load 2.5-3.0 μ l of each reaction on the gel.

b) Sequencing Protocol Using Direct Incorporation

i) Extension/Termination Reactions

The following procedure requires about 500 fmol of plasmid templates and about 40 fmol of PCR product. The end-labeled primer protocol (section a) is recommended for PCR templates. This procedure is not recommended for the sequencing of lambda templates.

For each set of sequencing reactions, label four 0.5 ml microcentrifuge tubes (G, A, T, C). Add 2 μ l of the appropriate d/ddNTP Mix to each tube. Cap the tubes and store on ice or at 4°C until needed. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 500 fmol template DNA (approx. 1 μ g of a 3-5 kb template); 3.0 pmol primer (approx. 25 ng of a 24 mer); 0.5 μ l [α -³⁵S]dATP (>1,000Ci/mmol, 10 μ Ci/ μ l) or [α -³²P]dATP (800Ci/mmol, 10 μ Ci/ μ l); 5 μ l *fmol*[®] Sequencing 5X Buffer and sterile H₂O to final volume of 16 μ l.

Add 1.0 μ l of Tne M284 (D323A,D389A) DNA Polymerase (5u/ μ l) to the primer/template mix. Mix briefly by pipetting up and down. Add 4 μ l of the enzyme/primer/template mix to the inside wall of each tube containing d/ddNTP Mix. Add one drop (approximately 20 μ l) of mineral oil to each tube and briefly spin in a microcentrifuge. Place the reaction tubes in a thermal cycler that has been preheated to 95°C and start the cycling program. The cycling profile chosen depends upon the characteristics of the primer used; see section a(i) above.

After the thermocycling program has been completed, add 3 μ l of *fmol*[®] Sequencing Stop Solution to the inside wall of each tube. Briefly spin in a microcentrifuge to terminate the reactions. Heat the reactions at 70°C for 2 minutes immediately before loading on a sequencing gel. Load 2.5-3.0 μ l of each reaction on the gel.

EXAMPLE 12¹²

Use Of Tne M284(D323A, D389A) Polymerase In Sanger Sequencing Protocols

The above examples described the use of the Tne M284(D323A, D389A) polymerase in thermal cycle sequencing protocols. The Tne M284(D323A, D389A) Polymerase may also be used in traditional Sanger sequencing protocols.

If a double stranded DNA template is used, the template is first denatured using alkali as follows. Four micrograms (approximately 2 pmol) of supercoiled plasmid DNA is added to a microcentrifuge tube and deionized H₂O is added to a final volume

of 18 μ l. Two microliters of 2 M NaOH, 2 mM EDTA is added and the mixture is incubated for 5 minutes at room temperature. To neutralize the reaction, add 8 μ l of 5M ammonium acetate, pH 7.5, and vortex. Add 112 μ l of 100% ethanol and vortex. Centrifuge the tube for 10 minutes at top speed in a microcentrifuge. Decant the supernatant. Wash the pellet with 1ml of 70% ethanol and centrifuge for 1 minute. Remove the supernatant and dry the pellet. Resuspend the dried pellet in 18 μ l of distilled water for sequencing. Proceed to either section i) or ii) depending on whether an end-labeled primer is employed or whether radiolabeled nucleotides are employed in the sequencing reaction.

a) Sequencing Protocol Using An End-Labeled Primer

The primer is end labeled using the protocol described in Example 11 (a)(i). The template and primer are annealed as follows. When a single-stranded DNA template is employed the following reaction is used. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 0.8 pmol ssDNA (approx. 2 μ g of an M13 template); 5.0 μ l of 5X *Taq* DNA Polymerase [250 mM Tris-HCL (pH 9.0 at 25°C); 50 mM MgCl₂]; 1.0 μ l labeled primer (1pmol); sterile dH₂O to a final volume of 25 μ l. Incubate at 37°C for 10 minutes. During the incubation, prepare the extension/termination reaction tubes as described in section c) below.

When a double-stranded plasmid is used as the template, the following reaction is used. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 1.6 pmol denatured plasmid dsDNA (approx. 4 μ g of a 3-5kb template); 5.0 μ l *Taq* DNA Polymerase 5X Buffer; 2.0 μ l labeled primer (2pmol) and sterile dH₂O to a final volume of 25 μ l. Incubate at 37°C for 10 minutes. During the incubation, prepare the extension/termination reaction tubes as described in section c) below.

b) Extension/Termination Reactions

For each set of sequencing reactions, label four microcentrifuge tubes (G,A,T and C) and add 1 μ l of the 8X *Tne* optimized d/ddNTP Mix (see Table 12 for components of the 8X mixes) to each tube. Cap the tubes and store on ice or at 4°C until needed. Add 1 μ l of *Tne* M284 (D323A,D389A)DNA Polymerase (5 u/ μ l) to the annealed primer/template mix (prepared as described above) and mix briefly by pipetting up and down.

Add 6 μ l of the enzyme/primer/template mix to each of the four tubes containing the d/ddNTP Mixes. Mix briefly by pipetting up and down. A brief spin may be needed to ensure that no liquid is left on the tube walls. Incubate at 70°C for 15 minutes. Add 4 μ l of Stop Solution to each tube and set at room temperature. Heat the reactions to $\geq 70^\circ\text{C}$ for 2-5 minutes before loading the sequencing gel. Load 2.5-3.0 μ l of each reaction on the gel (6% denaturing polyacrylamide). Following electrophoresis of the sequencing gel, the gel is exposed to X-ray film to generate an autoradiograph.

If the extension products seen on the autoradiograph are too short, the ddNTP concentrations should be lowered and conversely if the extension products are all skewed to high molecular weight products, the ddNTP concentrations should be raised.

TABLE 12

8X Nucleotide Mix Formulation for Sanger
Sequencing Using *Tne* M284(D323A, D389A) Polymerase

	G	A	T	C
Nucleotide Component	Nucleotide Mix	Nucleotide Mix	Nucleotide Mix	Nucleotide Mix
ddGTP	20 μ M	-	-	-
ddATP	-	50 μ M	-	-
ddTTP	-	-	75 μ M	-
ddCTP	-	-	-	25 μ M
7-deaza dGTP	30 μ M	250 μ M	250 μ M	250 μ M
dATP	250 μ M	30 μ M	250 μ M	250 μ M
dTTP	250 μ M	250 μ M	30 μ M	250 μ M
dCTP	250 μ M	250 μ M	250 μ M	30 μ M

c) Sequencing Protocol Using Direct Incorporation

i) Annealing The Template And Primer

When a single-stranded DNA template is employed the following reaction is used to anneal the template and primer. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 0.8 pmol ssDNA (approx. 2 μ g of an M13 template); 1.0 pmol primer (approx. 8ng of a 24mer); 5.0 μ l *Taq* DNA Polymerase 5X Buffer; 2.0 μ l Extension/Labeling Mix [7.5 μ M each of dGTP, dTTP and dCTP]; sterile dH₂O to a final volume of 25 μ l. Incubate at 37°C for 10 minutes. During the incubation, prepare the nucleotide tubes for the termination reaction as described in section iii, below.

When a double-stranded DNA template is employed the following reaction is used to anneal the template and primer. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube: 1.6pmol denatured plasmid dsDNA (approx. 4 μ g of a 3-5kb template); 2pmol primer (approx. 16ng of a 24mer); 5.0 μ l *Taq* DNA Polymerase 5X Buffer; 2.0 μ l Extension/Labeling Mix; sterile dH₂O to a final volume of 25 μ l. Incubate at 37°C for 10 minutes. During the incubation, prepare the nucleotide tubes for the termination reaction as described in section iii, below.

ii) Extension/Labeling Reaction

Add 0.5 μ l of [α -³⁵S]dATP (1,000Ci/mmol, approximately 10 μ Ci/ μ l) or 0.5 μ l of [α -³²P]dATP (800Ci/mmol, approximately 10 μ Ci/ μ l) to the annealed primer/template mixture. Add 1 μ l of *Tne* M284 (D323A,D389A) DNA Polymerase (5u/ μ l) and mix briefly by pipetting up and down. Incubate at 37°C for 5 minutes.

iii) Termination Reaction

For each set of sequencing reactions, label four microcentrifuge tubes (G,A,T,C) and add 1 μ l of the 8X *Tne* optimized d/ddNTP Mix to each tube (see Table 12 above). Store on ice or at 4°C until just before completion of the extension/labeling reaction. When the extension/labeling reaction is complete, aliquot

6 μ l to each tube (G,A,T,C) containing d/ddNTP Mix. Mix briefly by pipetting up and down. A brief spin may be needed to ensure that no liquid is left on the tube walls. Incubate at 70°C for 15 minutes. Add 4 μ l of Stop Solution to each tube and store at -20°C. Heat the reactions to $\geq 70^\circ\text{C}$ for 2-5 minutes immediately before loading on a sequencing gel. Load 2.5-3.0 μ l of each reaction on the gel. Following electrophoresis of the sequencing gel, the gel is exposed to X-ray film to generate an autoradiograph.

If the extension products seen on the autoradiograph are too short, the ddNTP concentrations should be lowered and conversely if the extension products are all skewed to high molecular weight products, the ddNTP concentrations should be raised.

EXAMPLE 12

Fidelity Of *Tne* DNA Polymerases

The fidelity of the *Tne* polymerases (full-length and modified forms) is measured using a PCR fidelity assay. This assay is based on the amplification, circularization, and transformation of the pUC19 derivative pLACIQ, which contains a functional *lacI*^q allele [Frey and Suppmann, Biochemica 2:8 (1995)]. PCR-derived mutations in *lacI* result in a de-repression of the expression of *lacZ* α and subsequent formation of a functional β -galactosidase enzyme, which can be easily detected on X-Gal indicator plates.

a) Construction Of pLACIQ

The truncated *lacI* gene present in pUC19 is replaced by a functional copy of *lacI*^q. pUC19 (GibcoBRL) is digested with *Pvu*II and *Afl*III and the 180 bp *Pvu*II-*Afl*III fragment of pUC19 is replaced by a 1189 bp DNA fragment encoding *lacI*^q. The 1189 bp *lacI*^q fragment is created by PCR amplification of residues 2972-4142 of pTrc 99 A (Pharmacia). The following primer pair is used in the PCR: 5'-CATGCCATGGCATGCATTTACGTTGACACCA-3' (SEQ ID NO:48) and

5'-TCCCCCGGGTTGCGCTCACTGCCCCGCTTTCCAGT-3' (SEQ ID NO:49). The oligonucleotide of SEQ ID NO:48 contains a *Nco*I recognition site and the oligonucleotide of SEQ ID NO:49 contains a *Sma*I recognition site. The PCR is performed using 18 cycles of denaturation at 94°C for 30 sec; annealing at 57°C for 30 sec and extension at 72°C for 4 min. The PCR is performed using *Pfu* DNA polymerase (Stratagene) in the buffer recommended by the manufacturer. The PCR products are digested with *Nco*I and *Sma*I to generate a 1189 bp fragment having a blunt end (compatible with *Pvu*II ends) and a 5' overhanging end compatible with the *A*/III digested end of pUC19. The 1189 bp fragment is ligated into the digested pUC19 using standard techniques.

The ligation products are used to transform the α -complementing *E. coli* strain DH5 α (GibcoBRL) and the desired plasmid, termed pLACIQ (3695 bp), is isolated using standard techniques. DH5 α cells containing pLACIQ produce white (LACI⁺) colonies on LB plates containing ampicillin (100 μ g/ml) and X-Gal (0.004% w/v).

b) The PCR Fidelity Assay

The template used in the PCR fidelity assay is prepared as follows. pLACIQ is linearized by digestion with *Dra* II. A typical PCR reaction contains 5 or 10 ng of linearized, gel-purified plasmid DNA.

The following primers are used in the PCR fidelity assay to amplify the *lacI*^a sequences of pLACIQ; both PCR primers used have *Cla* I cleavage sites at their 5' ends: 5'-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3' (SEQ ID NO:50) and 5'-AGCTTATCGATAAGCGATGCCGGGAGCAGACAAGC-3' (SEQ ID NO:51). The length of the resulting PCR product is 3,561 bp.

The PCRs which employ the *Tne* DNA polymerases or *Ultma* DNA polymerase are performed using the following buffer [10 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.002% Tween 20, 1.5 mM MgCl₂ and 40 μ M all four dNTPs]. PCRs which employ nTaq DNA polymerase are performed using the following buffer [10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100

and 140 μ M all four dNTPs]. All reactions contain 5-10 ng of template, 20 pmol of each primer and 2.5 units of polymerase (all polymerases are assayed using the assay described in Example 5 to ensure the same amount of activity is used for each enzyme tested). The following cycling conditions are used: denaturation for 10 sec at 94°C; annealing for 30 sec at 57°C and extension for 4 min at 72°C for 18 cycles.

Following the PCR, the amplification products are PEG-precipitated as follows. The PCR amplification mixtures are frozen at -20°C and the mineral oil is removed by rinsing twice with cold chloroform. The mixtures are then thawed and 10 μ g of glycogen and 1/2 volume of 30% PEG 3350/1.5 M NaCl is added. The mixture is allowed to stand for a minimum of 1 hr at 0-4°C. The mixture is then centrifuged in a microcentrifuge for 8 min and the supernatant is removed. The pellet is then rinsed with 75% ethanol and dried. The DNA is then digested with *Cla*I and the digested DNA is purified by gel electrophoresis. The purified DNA is then ligated to recircularize the plasmid in a reaction containing less than or equal to 30 ng DNA.

The resulting PCR-derived plasmids are transformed into competent *E. coli* DH5 α and plated on LB Amp100 X-Gal plates [LB plates containing 100 μ g/ml ampicillin and 0.004% X-Gal (w/v)]. After incubation overnight at 37°C, blue and white colonies are counted. The error rate (f) per bp is calculated using the rearranged equation published by Keohavong and Thilly [Proc. Natl. Acad. Sci. USA 86:9253 (1989): $f = \ln F / d \times b$ bp; where F is the fraction of white colonies: $F = \text{white (LACI}^+) / \text{total colony number}$; 2^d is the number of DNA duplications: $2^d = \text{output DNA} / \text{input DNA}$; and b is the effective target size of the (1080 bp) *lacI* gene, which is 349 bp according to Provost *et al.* [Mut. Research 288:133 (1993)]; there are 349 phenotypically identified (by color screening) single-based substitutions (nonsense and mis-sense) at 179 codons (approximately 50% of the coding region) within the *lacI* gene [Provost *et al.*, *supra*]. Frameshift errors, which may occur at every position in the 1080 bp open reading frame of *lacI*, are not taken into account.

A religation control is prepared as follows. Fifty nanograms of *Dra* II-linearized, gel-purified pLACIQ DNA is religated, and an aliquot of the ligation

reaction is transformed into DH5 α . After incubation overnight, the number of growing colonies (0.027%) showing a blue (LACI⁻) phenotype on LB Amp X-Gal plates is measured to assess the rate of the formation of concatameric ligation products (with subsequent intramolecular recombination in *E. coli* that eliminates an additional origin of replication), which seems to be a very rare event. Restriction analysis of PCR-derived plasmids isolated from blue colonies is also performed to confirm that the LACI⁻ phenotype originates in PCR-derived mutations of *lacI*, but not in deleterious recombination events after transformation of the ligated DNA in DH5 α .

From the above, it is clear that the enzymes of the present invention provide thermostable DNA polymerase having novel features. In particular, these enzymes provide superior polymerases for use in PCR applications. In addition, these polymerases have higher affinities for ddNTPs which results in significant cost savings for users of chain termination sequencing protocols.

Other modifications of the embodiments of the invention described above that are obvious to those of ordinary skill in the areas of molecular biology, biochemistry and related disciplines are intended to be within the scope of the accompanying claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: MODIFIED THERMOPHILIC DNA POLYMERASES
FROM THERMOTOGA NEAPOLITANA

(iii) NUMBER OF SEQUENCES: 51

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: CARROLL, PETER G.
(B) REGISTRATION NUMBER: 32,837
(C) REFERENCE/DOCKET NUMBER: PRMG-01175

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 705-8410
(B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2682 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..2682

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG GCG AGA CTA TTT CTC TTT GAT GGC ACA GCC CTG GCC TAC AGG GCA
Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala
1 5 10 15

48

TAT Tyr	TAC Tyr	GCC Ala	CTC Leu	GAC Asp	AGA Arg	TCC Ser	CTT Leu	TCC Ser	ACA Thr	TCC Ser	ACA Thr	GGA Gly	ATT Ile	CCA Pro	ACG Thr	96
			20					25					30			
AAC Asn	GCC Ala	GTC Val	TAT Tyr	GGC Gly	GTT Val	GCC Ala	AGG Arg	ATG Met	CTC Leu	GTT Val	AAA Lys	TTC Phe	ATA Ile	AAG Lys	GAA Glu	144
		35					40					45				
CAC His	ATT Ile	ATA Ile	CCC Pro	GAA Glu	AAG Lys	GAC Asp	TAC Tyr	GCG Ala	GCT Ala	GTG Val	GCC Ala	TTC Phe	GAC Asp	AAG Lys	AAG Lys	192
	50					55					60					
GCA Ala	GCG Ala	ACG Thr	TTC Phe	AGA Arg	CAC His	AAA Lys	CTG Leu	CTC Leu	GAA Glu	GCG Ala	TAC Tyr	AAG Lys	GCG Ala	CAA Gln	AGG Arg	240
65					70				75						80	
CCA Pro	AAG Lys	ACG Thr	CCG Pro	GAT Asp	CTT Leu	CTA Leu	GTT Val	CAG Gln	CAG Gln	CTA Leu	CCT Pro	TAC Tyr	ATC Ile	AAG Lys	CGG Arg	288
				85				90						95		
CTG Leu	ATA Ile	GAA Glu	GCT Ala	CTT Leu	GGT Gly	TTC Phe	AAA Lys	GTG Val	CTG Leu	GAG Glu	CTG Leu	GAA Glu	GGA Gly	TAC Tyr	GAA Glu	336
			100					105					110			
GCA Ala	GAC Asp	GAT Asp	ATC Ile	ATC Ile	GCC Ala	ACG Thr	CTT Leu	GCA Ala	GTC Val	AAG Lys	GGC Gly	TGC Cys	ACG Thr	TTT Phe	TTT Phe	384
		115					120					125				
GAT Asp	GAG Glu	ATT Ile	TTC Phe	ATA Ile	ATA Ile	ACC Thr	GGT Gly	GAC Asp	AAG Lys	GAT Asp	ATG Met	CTT Leu	CAA Gln	CTT Leu	GTA Val	432
	130					135				140						
AAC Asn	GAG Glu	AAG Lys	ATA Ile	AAG Lys	GTC Val	TGG Trp	AGA Arg	ATC Ile	GTC Val	AAG Lys	GGG Gly	ATA Ile	TCG Ser	GAT Asp	CTT Leu	480
145					150					155					160	
GAG Glu	CTT Leu	TAC Tyr	GAT Asp	TCG Ser	AAA Lys	AAG Lys	GTG Val	AAA Lys	GAA Glu	AGA Arg	TAC Tyr	GGT Gly	GTG Val	GAA Glu	CCA Pro	528
				165				170					175			
CAT His	CAG Gln	ATA Ile	CCG Pro	GAT Asp	CTT Leu	CTA Leu	GCA Ala	CTG Leu	ACG Thr	GGA Gly	GAC Asp	GAG Glu	ATA Ile	GAC Asp	AAC Asn	576
			180					185					190			
ATT Ile	CCC Pro	GGT Gly	GTA Val	ACG Thr	GGA Gly	ATA Ile	GGT Gly	GAA Glu	AAG Lys	ACC Thr	GCT Ala	GTA Val	CAG Gln	CTT Leu	CTC Leu	624
		195					200					205				
GGC Gly	AAG Lys	TAC Tyr	AGA Arg	AAT Asn	CTT Leu	GAA Glu	GAC Asp	ATT Ile	CTG Leu	GAG Glu	CAT His	GCC Ala	CGT Arg	GAA Glu	CTC Leu	672
	210				215					220						
CCC Pro	CAG Gln	AGA Arg	GTG Val	AGA Arg	AAG Lys	GCT Ala	CTC Leu	TTG Leu	AGA Arg	GAC Asp	AGG Arg	GAA Glu	GTT Val	GCC Ala	ATC Ile	720
225					230					235					240	
CTC Leu	AGT Ser	AAA Lys	AAA Lys	CTT Leu	GCA Ala	ACT Thr	CTG Leu	GTG Val	ACG Thr	AAC Asn	GCA Ala	CCT Pro	GTT Val	GAA Glu	GTG Val	768
				245					250					255		

GAC Asp	TGG Trp	GAA Glu	GAG Glu	ATG Met	AAA Lys	TAC Tyr	AGA Arg	GGA Gly	TAC Tyr	GAC Asp	AAG Lys	AGA Arg	AAA Lys	CTA Leu	CTT Leu	816
260 265 270																
CCG Pro	ATA Ile	TTG Leu	AAA Lys	GAA Glu	CTG Leu	GAG Glu	TTT Phe	GCT Ala	TCC Ser	ATC Ile	ATG Met	AAG Lys	GAA Glu	CTT Leu	CAA Gln	864
275 280 285																
CTG Leu	TAC Tyr	GAA Glu	GAA Glu	GCA Ala	GAA Glu	CCC Pro	ACC Thr	GGA Gly	TAC Tyr	GAA Glu	ATC Ile	GTG Val	AAG Lys	GAT Asp	CAT His	912
290 295 300																
AAG Lys	ACC Thr	TTC Phe	GAA Glu	GAT Asp	CTC Leu	ATC Ile	GAA Glu	AAG Lys	CTG Leu	AAG Lys	GAG Glu	GTT Val	CCA Pro	TCT Ser	TTT Phe	960
305 310 315 320																
GCC Ala	CTG Leu	GAC Asp	CTT Leu	GAA Glu	ACG Thr	TCC Ser	TCC Ser	CTT Leu	GAC Asp	CCG Pro	TTC Phe	AAC Asn	TGT Cys	GAG Glu	ATA Ile	1008
325 330 335																
GTC Val	GGC Gly	ATC Ile	TCC Ser	GTG Val	TCG Ser	TTC Phe	AAA Lys	CCG Pro	AAA Lys	ACA Thr	GCT Ala	TAT Tyr	TAC Tyr	ATT Ile	CCA Pro	1056
340 345 350																
CTT Leu	CAT His	CAC His	AGA Arg	AAC Asn	GCC Ala	CAG Gln	AAT Asn	CTT Leu	GAT Asp	GAA Glu	ACA Thr	CTG Leu	GTG Val	CTG Leu	TCG Ser	1104
355 360 365																
AAG Lys	TTG Leu	AAA Lys	GAG Glu	ATC Ile	CTC Leu	GAA Glu	GAC Asp	CCG Pro	TCT Ser	TCG Ser	AAG Lys	ATT Ile	GTG Val	GGT Gly	CAG Gln	1152
370 375 380																
AAC Asn	CTG Leu	AAG Lys	TAC Tyr	GAC Asp	TAC Tyr	AAG Lys	GTT Val	CTT Leu	ATG Met	GTA Val	AAG Lys	GGT Gly	ATA Ile	TCG Ser	CCA Pro	1200
385 390 395 400																
GTT Val	TAT Tyr	CCG Pro	CAT His	TTT Phe	GAC Asp	ACG Thr	ATG Met	ATA Ile	GCT Ala	GCA Ala	TAT Tyr	TTG Leu	CTG Leu	GAG Glu	CCA Pro	1248
405 410 415																
AAC Asn	GAG Glu	AAA Lys	AAA Lys	TTC Phe	AAT Asn	CTC Leu	GAA Glu	GAT Asp	CTG Leu	TCT Ser	TTG Leu	AAA Lys	TTT Phe	CTC Leu	GGA Gly	1296
420 425 430																
TAC Tyr	AAA Lys	ATG Met	ACG Thr	TCT Ser	TAT Tyr	CAG Gln	GAA Glu	CTG Leu	ATG Met	TCG Ser	TTT Phe	TCC Ser	TCA Ser	CCA Pro	CTT Leu	1344
435 440 445																
TTT Phe	GGT Gly	TTC Phe	AGC Ser	TTT Phe	GCG Ala	GAT Asp	GTT Val	CCG Pro	GTA Val	GAC Asp	AAG Lys	GCT Ala	GCG Ala	AAC Asn	TAC Tyr	1392
450 455 460																
TCC Ser	TGC Cys	GAG Glu	GAT Asp	GCA Ala	GAC Asp	ATC Ile	ACT Thr	TAT Tyr	AGG Arg	CTC Leu	TAC Tyr	AAG Lys	ATA Ile	CTC Leu	AGC Ser	1440
465 470 475 480																
ATG Met	AAG Lys	CTC Leu	CAT His	GAA Glu	GCG Ala	GAA Glu	CTT Leu	GAG Glu	AAC Asn	GTC Val	TTC Phe	TAC Tyr	AGG Arg	ATA Ile	GAG Glu	1488
485 490 495																

ATG CCG CTT GTG AAC GTT CTT GCA CGC ATG GAA TTG AAC GGG GTG TAT Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr 500 505 510	1536
GTG GAC ACA GAA TTC CTG AAA AAG CTC TCG GAG GAG TAC GGC AAA AAG Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys 515 520 525	1584
CTC GAG GAA CTG GCC GAA AAA ATC TAC CAG ATA GCA GGA GAG CCC TTC Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe 530 535 540	1632
AAC ATC AAT TCT CCA AAA CAG GTT TCA AAG ATC CTT TTT GAG AAG CTG Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile Leu Phe Glu Lys Leu 545 550 555 560	1680
GGA ATA AAA CCC CGT GGA AAA ACG ACA AAA ACA GGA GCG TAC TCT ACC Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Ala Tyr Ser Thr 565 570 575	1728
AGG ATA GAG GTG TTG GAA GAG ATA GCG AAT GAG CAC GAG ATA GTA CCC Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val Pro 580 585 590	1776
CTC ATT CTC GAG TAC AGA AAG ATC CAG AAA CTG AAA TCG ACC TAC ATA Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr Ile 595 600 605	1824
GAC ACC CTT CCG AAA CTT GTG AAC CCG AAA ACC GGA AGA ATT CAT GCA Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Ile His Ala 610 615 620	1872
TCT TTC CAC CAG ACG GGT ACC GCC ACT GGC AGG TTG AGT AGC AGT GAT Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp 625 630 635 640	1920
CCA AAT CTT CAG AAT CTT CCG ACA AAG AGC GAA GAG GGA AAA GAA ATT Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile 645 650 655	1968
AGA AAA GCG ATT GTG CCC CAG GAT CCA GAC TGG TGG ATC GTC AGT GCG Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser Ala 660 665 670	2016
GAT TAT TCC CAA ATA GAA CTC AGA ATC CTC GCT CAT CTC AGT GGT GAT Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp 675 680 685	2064
GAG AAC CTT GTG AAG GCC TTC GAG GAG GGC ATC GAT GTG CAC ACC TTG Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu 690 695 700	2112
ACT GCC TCC AGG ATC TAC AAC GTA AAG CCA GAA GAA GTG AAC GAA GAA Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu Glu 705 710 715 720	2160
ATG CGA CGG GTT GGA AAG ATG GTG AAC TTC TCT ATA ATA TAC GGT GTC Met Arg Arg Val Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly Val 725 730 735	2208

ACA CCG TAC GGT CTT TCT GTG AGA CTT GGA ATA CCG GTT AAA GAA GCA Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile Pro Val Lys Glu Ala 740 745 750	2256
GAA AAG ATG ATT ATC AGC TAT TTC ACA CTG TAT CCA AAG GTG CGA AGC Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr Pro Lys Val Arg Ser 755 760 765	2304
TAC ATC CAG CAG GTT GTT GCA GAG GCA AAA GAG AAG GGC TAC GTC AGG Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu Lys Gly Tyr Val Arg 770 775 780	2352
ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG CTC ATG GCA AGG GAC Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg Asp 785 790 795 800	2400
AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA ATA AAC ACC CCC ATT Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro Ile 805 810 815	2448
CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT ATG ATA GAT ATA GAC Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Asp Ile Asp 820 825 830	2496
GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA ATG ATC ATT CAG GTT Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg Met Ile Ile Gln Val 835 840 845	2544
CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG GAA AAA GAA GAA CTA His Asp Glu Leu Val Phe Glu Val Pro Asp Glu Glu Lys Glu Glu Leu 850 855 860	2592
GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG GTG AAA CTC TCT GTG Val Asp Leu Val Lys Asn Lys Met Thr Asn Val Val Lys Leu Ser Val 865 870 875 880	2640
CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC TGG TCT TG Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser Trp Ser 885 890	2682

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 893 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala 1 5 10 15
Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr 20 25 30
Asn Ala Val Tyr Gly Val Ala Arg Met Leu Val Lys Phe Ile Lys Glu 35 40 45
His Ile Ile Pro Glu Lys Asp Tyr Ala Ala Val Ala Phe Asp Lys Lys 50 55 60

Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro
405 410 415

Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly
420 425 430

Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Ser Pro Leu
435 440 445

Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp Lys Ala Ala Asn Tyr
450 455 460

Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Ile Leu Ser
465 470 475 480

Met Lys Leu His Glu Ala Glu Leu Glu Asn Val Phe Tyr Arg Ile Glu
485 490 495

Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr
500 505 510

Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys
515 520 525

Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe
530 535 540

Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile Leu Phe Glu Lys Leu
545 550 555 560

Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Ala Tyr Ser Thr
565 570 575

Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val Pro
580 585 590

Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr Ile
595 600 605

Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Ile His Ala
610 615 620

Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp
625 630 635 640

Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile
645 650 655

Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser Ala
660 665 670

Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp
675 680 685

Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu
690 695 700

Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu Glu
705 710 715 720

Met Arg Arg Val Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly Val
725 730 735

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGCCAGGGC TGTGCCATCA AAGAGAAATA GTCTCGCCA

39

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TATGGCGAGA CTATTCTCT TTGATGGCAC AGCCCTGGCC TACA

44

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1833 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu
1 5 10 15

48

ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys
20 25 30

96

GAG GTT CCA TCT TTT GCC CTG GAC CTT GAA ACG TCC TCC CTT GAC CCG
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro
35 40 45

144

TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr
50 55 60

192

GCT Ala 65	TAT Tyr	TAC Tyr	ATT Ile	CCA Pro	CTT Leu 70	CAT His	CAC His	AGA Arg	AAC Asn	GCC Ala 75	CAG Gln	AAT Asn	CTT Leu	GAT Asp	GAA Glu 80	240
ACA Thr	CTG Leu	GTG Val	CTG Leu	TCG Ser 85	AAG Lys	TTG Leu	AAA Lys	GAG Glu	ATC Ile 90	CTC Leu	GAA Glu	GAC Asp	CCG Pro	TCT Ser 95	TCG Ser	288
AAG Lys	ATT Ile	GTG Val	GGT Gly 100	CAG Gln	AAC Asn	CTG Leu	AAG Lys	TAC Tyr 105	GAC Asp	TAC Tyr	AAG Lys	GTT Val	CTT Leu 110	ATG Met	GTA Val	336
AAG Lys	GGT Gly 115	ATA Ile	TCG Ser	CCA Pro	GTT Val	TAT Tyr	CCG Pro 120	CAT His	TTT Phe	GAC Asp	ACG Thr	ATG Met 125	ATA Ile	GCT Ala	GCA Ala	384
TAT Tyr 130	TTG Leu	CTG Leu	GAG Glu	CCA Pro	AAC Asn	GAG Glu 135	AAA Lys	AAA Lys	TTC Phe	AAT Asn	CTC Leu 140	GAA Glu	GAT Asp	CTG Leu	TCT Ser	432
TTG Leu 145	AAA Lys	TTT Phe	CTC Leu	GGA Gly 150	TAC Tyr 150	AAA Lys	ATG Met	ACG Thr	TCT Ser	TAT Tyr 155	CAG Gln	GAA Glu	CTG Leu	ATG Met	TCG Ser 160	480
TTT Phe	TCC Ser	TCA Ser	CCA Pro	CTT Leu 165	TTT Phe	GGT Gly	TTC Phe	AGC Ser	TTT Phe 170	GCG Ala	GAT Asp	GTT Val	CCG Pro	GTA Val 175	GAC Asp	528
AAG Lys	GCT Ala	GCG Ala	AAC Asn 180	TAC Tyr	TCC Ser	TGC Cys	GAG Glu	GAT Asp 185	GCA Ala	GAC Asp	ATC Ile	ACT Thr	TAT Tyr 190	AGG Arg	CTC Leu	576
TAC Tyr 195	AAG Lys	ATA Ile	CTC Leu	AGC Ser	ATG Met	AAG Lys	CTC Leu 200	CAT His	GAA Glu	GCG Ala	GAA Glu	CTT Leu 205	GAG Glu	AAC Asn	GTC Val	624
TTC Phe 210	TAC Tyr	AGG Arg	ATA Ile	GAG Glu	ATG Met	CCG Pro 215	CTT Leu	GTG Val	AAC Asn	GTT Val 220	CTT Leu	GCA Ala	CGC Arg	ATG Met	GAA Glu	672
TTG Leu 225	AAC Asn	GGG Gly	GTG Val	TAT Tyr	GTG Val 230	GAC Asp	ACA Thr	GAA Glu	TTC Phe	CTG Leu 235	AAA Lys	AAG Lys	CTC Leu	TCG Ser	GAG Glu 240	720
GAG Glu	TAC Tyr	GGC Gly	AAA Lys	AAG Lys 245	CTC Leu	GAG Glu	GAA Glu	CTG Leu	GCC Ala 250	GAA Glu	AAA Lys	ATC Ile	TAC Tyr	CAG Gln 255	ATA Ile	768
GCA Ala	GGA Gly	GAG Glu	CCC Pro 260	TTC Phe	AAC Asn	ATC Ile	AAT Asn	TCT Ser 265	CCA Pro	AAA Lys	CAG Gln	GTT Val	TCA Ser 270	AAG Lys	ATC Ile	816
CTT Leu	TTT Phe	GAG Glu	AAG Lys	CTG Leu	GGA Gly	ATA Ile	AAA Lys 280	CCC Pro	CGT Arg	GGA Gly	AAA Lys	ACG Thr 285	ACA Thr	AAA Lys	ACA Thr	864
GGA Gly 290	GCG Ala	TAC Tyr	TCT Ser	ACC Thr	AGG Arg	ATA Ile	GAG Glu 295	GTG Val	TTG Leu	GAA Glu	GAG Glu	ATA Ile	GCG Ala	AAT Asn	GAG Glu	912

Phe	Ser	Ser	Pro	Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp	
				165					170					175		
Lys	Ala	Ala	Asn	Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu	
			180					185					190			
Tyr	Lys	Ile	Leu	Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val	
		195					200					205				
Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu	
	210					215					220					
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu	
225					230					235					240	
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile	
			245						250					255		
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile	
			260					265					270			
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr	
		275					280					285				
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu	
	290					295					300					
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu	
305					310					315					320	
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr	
			325						330					335		
Gly	Arg	Ile	His	Ala	Ser	Phe	His	Gln	Thr	Gly	Thr	Ala	Thr	Gly	Arg	
			340					345					350			
Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln	Asn	Leu	Pro	Thr	Lys	Ser	Glu	
		355					360					365				
Glu	Gly	Lys	Glu	Ile	Arg	Lys	Ala	Ile	Val	Pro	Gln	Asp	Pro	Asp	Trp	
	370					375					380					
Trp	Ile	Val	Ser	Ala	Asp	Tyr	Ser	Gln	Ile	Glu	Leu	Arg	Ile	Leu	Ala	
385					390					395					400	
His	Leu	Ser	Gly	Asp	Glu	Asn	Leu	Val	Lys	Ala	Phe	Glu	Glu	Gly	Ile	
			405						410					415		
Asp	Val	His	Thr	Leu	Thr	Ala	Ser	Arg	Ile	Tyr	Asn	Val	Lys	Pro	Glu	
			420					425					430			
Glu	Val	Asn	Glu	Glu	Met	Arg	Arg	Val	Gly	Lys	Met	Val	Asn	Phe	Ser	
		435					440					445				
Ile	Ile	Tyr	Gly	Val	Thr	Pro	Tyr	Gly	Leu	Ser	Val	Arg	Leu	Gly	Ile	
	450					455					460					
Pro	Val	Lys	Glu	Ala	Glu	Lys	Met	Ile	Ile	Ser	Tyr	Phe	Thr	Leu	Tyr	
465					470					475					480	
Pro	Lys	Val	Arg	Ser	Tyr	Ile	Gln	Gln	Val	Val	Ala	Glu	Ala	Lys	Glu	
			485						490					495		

Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln
 500 505 510
 Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala
 515 520 525
 Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala
 530 535 540
 Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg
 545 550 555 560
 Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu
 565 570 575
 Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val
 580 585 590
 Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
 595 600 605
 Trp Ser
 610

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCGAAAAGC TGACCATGGT TCCATCTTTT G

31

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1737 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1737

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATG GTT CCA TCT TTT GCC CTG GAC CTT GAA ACG TCC TCC CTT GAC CCG
 Met Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro
 1 5 10 15

48

TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA
 Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr
 20 25 30

96

CAC GAG ATA GTA CCC CTC ATT CTC GAG TAC AGA AAG ATC CAG AAA CTG His Glu Ile Val Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu 275 280 285	864
AAA TCG ACC TAC ATA GAC ACC CTT CCG AAA CTT GTG AAC CCG AAA ACC Lys Ser Thr Tyr Ile Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr 290 295 300	912
GGA AGA ATT CAT GCA TCT TTC CAC CAG ACG GGT ACC GCC ACT GGC AGG Gly Arg Ile His Ala Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg 305 310 315 320	960
TTG AGT AGC AGT GAT CCA AAT CTT CAG AAT CTT CCG ACA AAG AGC GAA Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu 325 330 335	1008
GAG GGA AAA GAA ATT AGA AAA GCG ATT GTG CCC CAG GAT CCA GAC TGG Glu Gly Lys Glu Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp 340 345 350	1056
TGG ATC GTC AGT GCG GAT TAT TCC CAA ATA GAA CTC AGA ATC CTC GCT Trp Ile Val Ser Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala 355 360 365	1104
CAT CTC AGT GGT GAT GAG AAC CTT GTG AAG GCC TTC GAG GAG GGC ATC His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile 370 375 380	1152
GAT GTG CAC ACC TTG ACT GCC TCC AGG ATC TAC AAC GTA AAG CCA GAA Asp Val His Thr Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu 385 390 395 400	1200
GAA GTG AAC GAA GAA ATG CGA CGG GTT GGA AAG ATG GTG AAC TTC TCT Glu Val Asn Glu Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser 405 410 415	1248
ATA ATA TAC GGT GTC ACA CCG TAC GGT CTT TCT GTG AGA CTT GGA ATA Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile 420 425 430	1296
CCG GTT AAA GAA GCA GAA AAG ATG ATT ATC AGC TAT TTC ACA CTG TAT Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr 435 440 445	1344
CCA AAG GTG CGA AGC TAC ATC CAG CAG GTT GTT GCA GAG GCA AAA GAG Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu 450 455 460	1392
AAG GGC TAC GTC AGG ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln 465 470 475 480	1440
CTC ATG GCA AGG GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala 485 490 495	1488
ATA AAC ACC CCC ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala 500 505 510	1536

ATG ATA GAT ATA GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA	1584
Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg	
515 520 525	
ATG ATC ATT CAG GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG	1632
Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu	
530 535 540	
GAA AAA GAA GAA CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG	1680
Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val	
545 550 555 560	
GTG AAA CTC TCT GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC	1728
Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser	
565 570 575	
TGG TCT TG	1737
Trp Ser	

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 578 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro	
1 5 10 15	
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
20 25 30	
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
35 40 45	
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
50 55 60	
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val	
65 70 75 80	
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
85 90 95	
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
100 105 110	
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	
115 120 125	
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp	
130 135 140	
Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu	
145 150 155 160	
Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val	
165 170 175	

Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu	
			180					185					190			
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu	
		195					200					205				
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile	
	210					215					220					
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile	
225					230					235					240	
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr	
				245					250					255		
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu	
			260					265					270			
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu	
		275					280					285				
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr	
	290					295					300					
Gly	Arg	Ile	His	Ala	Ser	Phe	His	Gln	Thr	Gly	Thr	Ala	Thr	Gly	Arg	
305					310					315					320	
Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln	Asn	Leu	Pro	Thr	Lys	Ser	Glu	
				325					330					335		
Glu	Gly	Lys	Glu	Ile	Arg	Lys	Ala	Ile	Val	Pro	Gln	Asp	Pro	Asp	Trp	
			340					345					350		-	
Trp	Ile	Val	Ser	Ala	Asp	Tyr	Ser	Gln	Ile	Glu	Leu	Arg	Ile	Leu	Ala	
		355					360					365				
His	Leu	Ser	Gly	Asp	Glu	Asn	Leu	Val	Lys	Ala	Phe	Glu	Glu	Gly	Ile	
	370					375					380					
Asp	Val	His	Thr	Leu	Thr	Ala	Ser	Arg	Ile	Tyr	Asn	Val	Lys	Pro	Glu	
385					390					395					400	
Glu	Val	Asn	Glu	Glu	Met	Arg	Arg	Val	Gly	Lys	Met	Val	Asn	Phe	Ser	
				405					410					415		
Ile	Ile	Tyr	Gly	Val	Thr	Pro	Tyr	Gly	Leu	Ser	Val	Arg	Leu	Gly	Ile	
			420					425					430			
Pro	Val	Lys	Glu	Ala	Glu	Lys	Met	Ile	Ile	Ser	Tyr	Phe	Thr	Leu	Tyr	
		435					440					445				
Pro	Lys	Val	Arg	Ser	Tyr	Ile	Gln	Gln	Val	Val	Ala	Glu	Ala	Lys	Glu	
	450					455					460					
Lys	Gly	Tyr	Val	Arg	Thr	Leu	Phe	Gly	Arg	Lys	Arg	Asp	Ile	Pro	Gln	
465					470					475					480	
Leu	Met	Ala	Arg	Asp	Lys	Asn	Thr	Gln	Ser	Glu	Gly	Glu	Arg	Ile	Ala	
				485					490					495		
Ile	Asn	Thr	Pro	Ile	Gln	Gly	Thr	Ala	Ala	Asp	Ile	Ile	Lys	Leu	Ala	
			500					505					510			

Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg
515 520 525

Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu
530 535 540

Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val
545 550 555 560

Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
565 570 575

Trp Ser

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGCCGTACAC CTCCGAGAGC

20

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCGTTTGGC TCCAGCAAAT ATGC

24

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTGCCCTGG AACTTGAAAC G

21

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1833 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GAA CTT GAA ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Glu Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GAC TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT	432
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
130 135 140	
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG	480
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	
145 150 155 160	
TTT TCC TCA CCA CTT TTT GGT TTC AGC TTT GCG GAT GTT CCG GTA GAC	528
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp	
165 170 175	

AAG GCT GCG AAC TAC TCC TGC GAG GAT GCA GAC ATC ACT TAT AGG CTC Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu 180 185 190	576
TAC AAG ATA CTC AGC ATG AAG CTC CAT GAA GCG GAA CTT GAG AAC GTC Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val 195 200 205	624
TTC TAC AGG ATA GAG ATG CCG CTT GTG AAC GTT CTT GCA CGC ATG GAA Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu 210 215 220	672
TTG AAC GGG GTG TAT GTG GAC ACA GAA TTC CTG AAA AAG CTC TCG GAG Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu 225 230 235 240	720
GAG TAC GGC AAA AAG CTC GAG GAA CTG GCC GAA AAA ATC TAC CAG ATA Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile 245 250 255	768
GCA GGA GAG CCC TTC AAC ATC AAT TCT CCA AAA CAG GTT TCA AAG ATC Ala Gly Glu Pro Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile 260 265 270	816
CTT TTT GAG AAG CTG GGA ATA AAA CCC CGT GGA AAA ACG ACA AAA ACA Leu Phe Glu Lys Leu Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr 275 280 285	864
GGA GCG TAC TCT ACC AGG ATA GAG GTG TTG GAA GAG ATA GCG AAT GAG Gly Ala Tyr Ser Thr Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu 290 295 300	912
CAC GAG ATA GTA CCC CTC ATT CTC GAG TAC AGA AAG ATC CAG AAA CTG His Glu Ile Val Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu 305 310 315 320	960
AAA TCG ACC TAC ATA GAC ACC CTT CCG AAA CTT GTG AAC CCG AAA ACC Lys Ser Thr Tyr Ile Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr 325 330 335	1008
GGA AGA ATT CAT GCA TCT TTC CAC CAG ACG GGT ACC GCC ACT GGC AGG Gly Arg Ile His Ala Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg 340 345 350	1056
TTG AGT AGC AGT GAT CCA AAT CTT CAG AAT CTT CCG ACA AAG AGC GAA Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu 355 360 365	1104
GAG GGA AAA GAA ATT AGA AAA GCG ATT GTG CCC CAG GAT CCA GAC TGG Glu Gly Lys Glu Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp 370 375 380	1152
TGG ATC GTC AGT GCG GAT TAT TCC CAA ATA GAA CTC AGA ATC CTC GCT Trp Ile Val Ser Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala 385 390 395 400	1200
CAT CTC AGT GGT GAT GAG AAC CTT GTG AAG GCC TTC GAG GAG GGC ATC His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile 405 410 415	1248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu
1 5 10 15
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys
20 25 30
Glu Val Pro Ser Phe Ala Leu Glu Leu Glu Thr Ser Ser Leu Asp Pro
35 40 45
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr
50 55 60
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu
65 70 75 80
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser
85 90 95
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val
100 105 110
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala
115 120 125
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser
130 135 140
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser
145 150 155 160
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp
165 170 175
Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu
180 185 190
Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val
195 200 205
Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu
210 215 220
Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu
225 230 235 240
Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile
245 250 255
Ala Gly Glu Pro Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile
260 265 270
Leu Phe Glu Lys Leu Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr
275 280 285
Gly Ala Tyr Ser Thr Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu
290 295 300
His Glu Ile Val Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu
305 310 315 320

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GACCTTGACA CGTCCTC

17

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1833 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GAC CTT GAC ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Asp Leu Asp Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GAC TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	

TAT	TTG	CTG	GAG	CCA	AAC	GAG	AAA	AAA	TTC	AAT	CTC	GAA	GAT	CTG	TCT	432
Tyr	Leu	Leu	Glu	Pro	Asn	Glu	Lys	Lys	Phe	Asn	Leu	Glu	Asp	Leu	Ser	
	130					135					140					
TTG	AAA	TTT	CTC	GGA	TAC	AAA	ATG	ACG	TCT	TAT	CAG	GAA	CTG	ATG	TCG	480
Leu	Lys	Phe	Leu	Gly	Tyr	Lys	Met	Thr	Ser	Tyr	Gln	Glu	Leu	Met	Ser	
145					150					155					160	
TTT	TCC	TCA	CCA	CTT	TTT	GGT	TTC	AGC	TTT	GCG	GAT	GTT	CCG	GTA	GAC	528
Phe	Ser	Ser	Pro	Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp	
				165					170					175		
AAG	GCT	GCG	AAC	TAC	TCC	TGC	GAG	GAT	GCA	GAC	ATC	ACT	TAT	AGG	CTC	576
Lys	Ala	Ala	Asn	Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu	
			180					185					190			
TAC	AAG	ATA	CTC	AGC	ATG	AAG	CTC	CAT	GAA	GCG	GAA	CTT	GAG	AAC	GTC	624
Tyr	Lys	Ile	Leu	Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val	
		195					200					205				
TTC	TAC	AGG	ATA	GAG	ATG	CCG	CTT	GTG	AAC	GTT	CTT	GCA	CGC	ATG	GAA	672
Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu	
	210					215					220					
TTG	AAC	GGG	GTG	TAT	GTG	GAC	ACA	GAA	TTC	CTG	AAA	AAG	CTC	TCG	GAG	720
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu	
225					230					235					240	
GAG	TAC	GGC	AAA	AAG	CTC	GAG	GAA	CTG	GCC	GAA	AAA	ATC	TAC	CAG	ATA	768
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile	
				245					250					255		
GCA	GGA	GAG	CCC	TTC	AAC	ATC	AAT	TCT	CCA	AAA	CAG	GTT	TCA	AAG	ATC	816
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile	
			260					265					270			
CTT	TTT	GAG	AAG	CTG	GGA	ATA	AAA	CCC	CGT	GGA	AAA	ACG	ACA	AAA	ACA	864
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr	
		275					280					285				
GGA	GCG	TAC	TCT	ACC	AGG	ATA	GAG	GTG	TTG	GAA	GAG	ATA	GCG	AAT	GAG	912
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu	
	290					295					300					
CAC	GAG	ATA	GTA	CCC	CTC	ATT	CTC	GAG	TAC	AGA	AAG	ATC	CAG	AAA	CTG	960
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu	
305					310					315					320	
AAA	TCG	ACC	TAC	ATA	GAC	ACC	CTT	CCG	AAA	CTT	GTG	AAC	CCG	AAA	ACC	1008
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr	
				325				330						335		
GGA	AGA	ATT	CAT													

TGG TCT TG
Trp Ser
610

1833

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 610 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu
 1 5 10 15
 Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys
 20 25 30
 Glu Val Pro Ser Phe Ala Leu Asp Leu Asp Thr Ser Ser Leu Asp Pro
 35 40 45
 Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr
 50 55 60
 Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu
 65 70 75 80
 Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser
 85 90 95
 Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val
 100 105 110
 Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala
 115 120 125
 Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser
 130 135 140
 Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser
 145 150 155 160
 Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp
 165 170 175
 Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu
 180 185 190
 Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val
 195 200 205
 Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu
 210 215 220
 Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu
 225 230 235 240
 Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile
 245 250 255

Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
 595 600 605

Trp Ser
 610

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 39 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TAAGTGATAT CTGCATCCTC GCAGGAGAAG TTCGCAGCC

39

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 39 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACAAGGCTGC GAACTTCTCC TGCGAGGATG CAGATATCA

39

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1833 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA
 Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu
 1 5 10 15

48

ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG
 Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys
 20 25 30

96

GAG GTT CCA TCT TTT GCC CTG GAC CTT GAA ACG TCC TCC CTT GAC CCG
 Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro
 35 40 45

144

TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr 50 55 60	192
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu 65 70 75 80	240
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser 85 90 95	288
AAG ATT GTG GGT CAG AAC CTG AAG TAC GAC TAC AAG GTT CTT ATG GTA Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val 100 105 110	336
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala 115 120 125	384
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser 130 135 140	432
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser 145 150 155 160	480
TTT TCC TCA CCA CTT TTT GGT TTC AGC TTT GCG GAT GTT CCG GTA GAC Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp 165 170 175	528
AAG GCT GCG AAC TTC TCC TGC GAG GAT GCA GAT ATC ACT TAT AGG CTC Lys Ala Ala Asn Phe Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu 180 185 190	576
TAC AAG ATA CTC AGC ATG AAG CTC CAT GAA GCG GAA CTT GAG AAC GTC Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val 195 200 205	624
TTC TAC AGG ATA GAG ATG CCG CTT GTG AAC GTT CTT GCA CGC ATG GAA Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu 210 215 220	672
TTG AAC GGG GTG TAT GTG GAC ACA GAA TTC CTG AAA AAG CTC TCG GAG Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu 225 230 235 240	720
GAG TAC GGC AAA AAG CTC GAG GAA CTG GCC GAA AAA ATC TAC CAG ATA Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile 245 250 255	768
GCA GGA GAG CCC TTC AAC ATC AAT TCT CCA AAA CAG GTT TCA AAG ATC Ala Gly Glu Pro Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile 260 265 270	816

CTT Leu	TTT Phe	GAG Glu 275	AAG Lys	CTG Leu	GGA Gly	ATA Ile 280	AAA Lys	CCC Pro	CGT Arg	GGA Gly	AAA Lys	ACG Thr 285	ACA Thr	AAA Lys	ACA Thr	864
GGA Gly	GCG Ala 290	TAC Tyr	TCT Ser	ACC Thr	AGG Arg	ATA Ile 295	GAG Glu	GTG Val	TTG Leu	GAA Glu	GAG Glu 300	ATA Ile	GCG Ala	AAT Asn	GAG Glu	912
CAC His 305	GAG Glu	ATA Ile	GTA Val	CCC Pro	CTC Leu 310	ATT Ile	CTC Leu	GAG Glu	TAC Tyr	AGA Arg 315	AAG Lys	ATC Ile	CAG Gln	AAA Lys	CTG Leu 320	960
AAA Lys	TCG Ser	ACC Thr	TAC Tyr	ATA Ile 325	GAC Asp	ACC Thr	CTT Leu	CCG Pro	AAA Lys 330	CTT Leu	GTG Val	AAC Asn	CCG Pro	AAA Lys 335	ACC Thr	1008
GGA Gly	AGA Arg	ATT Ile	CAT His 340	GCA Ala	TCT Ser	TTC Phe	CAC His	CAG Gln 345	ACG Thr	GGT Gly	ACC Thr	GCC Ala	ACT Thr 350	GGC Gly	AGG Arg	1056
TTG Leu	AGT Ser	AGC Ser 355	AGT Ser	GAT Asp	CCA Pro	AAT Asn	CTT Leu 360	CAG Gln	AAT Asn	CTT Leu	CCG Pro	ACA Thr 365	AAG Lys	AGC Ser	GAA Glu	1104
GAG Glu 370	GGA Gly	AAA Lys	GAA Glu	ATT Ile	AGA Arg	AAA Lys 375	GCG Ala	ATT Ile	GTG Val	CCC Pro	CAG Gln 380	GAT Asp	CCA Pro	GAC Asp	TGG Trp	1152
TGG Trp 385	ATC Ile	GTC Val	AGT Ser	GCG Ala	GAT Asp 390	TAT Tyr	TCC Ser	CAA Gln	ATA Ile	GAA Glu 395	CTC Leu	AGA Arg	ATC Ile	CTC Leu	GCT Ala 400	1200
CAT His	CTC Leu	AGT Ser	GGT Gly	GAT Asp 405	GAG Glu	AAC Asn	CTT Leu	GTG Val	AAG Lys 410	GCC Ala	TTC Phe	GAG Glu	GAG Glu	GGC Gly 415	ATC Ile	1248
GAT Asp	GTG Val	CAC His	ACC Thr 420	TTG Leu	ACT Thr	GCC Ala	TCC Ser	AGG Arg 425	ATC Ile	TAC Tyr	AAC Asn	GTA Val 430	AAG Lys	CCA Pro	GAA Glu	1296
GAA Glu	GTG Val	AAC Asn 435	GAA Glu	GAA Glu	ATG Met	CGA Arg	CGG Arg 440	GTT Val	GGA Gly	AAG Lys	ATG Met	GTG Val 445	AAC Asn	TTC Phe	TCT Ser	1344
ATA Ile 450	ATA Ile	TAC Tyr	GGT Gly	GTC Val	ACA Thr	CCG Pro 455	TAC Tyr	GGT Gly	CTT Leu	TCT Ser	GTG Val 460	AGA Arg	CTT Leu	GGA Gly	ATA Ile	1392
CCG Pro 465	GTT Val	AAA Lys	GAA Glu	GCA Ala	GAA Glu 470	AAG Lys	ATG Met	ATT Ile	ATC Ile	AGC Ser 475	TAT Tyr	TTC Phe	ACA Thr	CTG Leu	TAT Tyr 480	1440
CCA Pro	AAG Lys	GTG Val	CGA Arg	AGC Ser 485	TAC Tyr	ATC Ile	CAG Gln	CAG Gln	GTT Val 490	GTT Val	GCA Ala	GAG Glu	GCA Ala	AAA Lys 495	GAG Glu	1488
AAG Lys	GGC Gly	TAC Tyr	GTC Val	AGG Arg	ACT Thr	CTC Leu	TTT Phe	GGA Gly	AGA Arg	AAA Lys	AGA Arg	GAT Asp	ATT Ile	CCC Pro	CAG Gln	1536

CTC ATG GCA AGG GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala 515 520 525	1584
ATA AAC ACC CCC ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala 530 535 540	1632
ATG ATA GAT ATA GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg 545 550 555 560	1680
ATG ATC ATT CAG GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu 565 570 575	1728
GAA AAA GAA GAA CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val 580 585 590	1776
GTG AAA CTC TCT GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser 595 600 605	1824
TGG TCT TG Trp Ser 610	1833

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu 1 5 10 15
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys 20 25 30
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro 35 40 45
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr 50 55 60
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu 65 70 75 80
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser 85 90 95
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val 100 105 110
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala 115 120 125

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GAC CTT GAA ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GAC TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT	432
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
130 135 140	
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG	480
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	
145 150 155 160	
TTT TCC TCA CCA CTT TTT GGT TTC AGC TTT GCG GAT GTT CCG GTA GAC	528
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp	
165 170 175	
AAG GCT GCG AAC TAC TCC TGC GAG AAT GCT GAC ATC ACT TAT AGG CTC	576
Lys Ala Ala Asn Tyr Ser Cys Glu Asn Ala Asp Ile Thr Tyr Arg Leu	
180 185 190	
TAC AAG ATA CTC AGC ATG AAG CTC CAT GAA GCG GAA CTT GAG AAC GTC	624
Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val	
195 200 205	
TTC TAC AGG ATA GAG ATG CCG CTT GTG AAC GTT CTT GCA CGC ATG GAA	672
Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu	
210 215 220	
TTG AAC GGG GTG TAT GTG GAC ACA GAA TTC CTG AAA AAG CTC TCG GAG	720
Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu	
225 230 235 240	

CCA AAG GTG CGA AGC TAC ATC CAG CAG GTT GTT GCA GAG GCA AAA GAG	1488
Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu	
485 490 495	
AAG GGC TAC GTC AGG ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG	1536
Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln	
500 505 510	
CTC ATG GCA AGG GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA	1584
Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala	
515 520 525	
ATA AAC ACC CCC ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT	1632
Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala	
530 535 540	
ATG ATA GAT ATA GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA	1680
Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg	
545 550 555 560	
ATG ATC ATT CAG GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG	1728
Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu	
565 570 575	
GAA AAA GAA GAA CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG	1776
Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val	
580 585 590	
GTG AAA CTC TCT GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC	1824
Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser	
595 600 605	
TGG TCT TG	1833
Trp Ser	
610	

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GCC CTT GAA ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Ala Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GAC TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT	432
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
130 135 140	
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG	480
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	
145 150 155 160	
TTT TCC TCA CCA CTT TTT GGT TTC AGC TTT GCG GAT GTT CCG GTA GAC	528
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp	
165 170 175	
AAG GCT GCG AAC TAC TCC TGC GAG GAT GCA GAC ATC ACT TAT AGG CTC	576
Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu	
180 185 190	
TAC AAG ATA CTC AGC ATG AAG CTC CAT GAA GCG GAA CTT GAG AAC GTC	624
Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val	
195 200 205	
TTC TAC AGG ATA GAG ATG CCG CTT GTG AAC GTT CTT GCA CGC ATG GAA	672
Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu	
210 215 220	

TTG AAC GGG GTG TAT GTG GAC ACA GAA TTC CTG AAA AAG CTC TCG GAG Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu 225 230 235 240	720
GAG TAC GGC AAA AAG CTC GAG GAA CTG GCC GAA AAA ATC TAC CAG ATA Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile 245 250 255	768
GCA GGA GAG CCC TTC AAC ATC AAT TCT CCA AAA CAG GTT TCA AAG ATC Ala Gly Glu Pro Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile 260 265 270	816
CTT TTT GAG AAG CTG GGA ATA AAA CCC CGT GGA AAA ACG ACA AAA ACA Leu Phe Glu Lys Leu Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr 275 280 285	864
GGA GCG TAC TCT ACC AGG ATA GAG GTG TTG GAA GAG ATA GCG AAT GAG Gly Ala Tyr Ser Thr Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu 290 295 300	912
CAC GAG ATA GTA CCC CTC ATT CTC GAG TAC AGA AAG ATC CAG AAA CTG His Glu Ile Val Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu 305 310 315 320	960
AAA TCG ACC TAC ATA GAC ACC CTT CCG AAA CTT GTG AAC CCG AAA ACC Lys Ser Thr Tyr Ile Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr 325 330 335	1008
GGA AGA ATT CAT GCA TCT TTC CAC CAG ACG GGT ACC GCC ACT GGC AGG Gly Arg Ile His Ala Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg 340 345 350	1056
TTG AGT AGC AGT GAT CCA AAT CTT CAG AAT CTT CCG ACA AAG AGC GAA Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu 355 360 365	1104
GAG GGA AAA GAA ATT AGA AAA GCG ATT GTG CCC CAG GAT CCA GAC TGG Glu Gly Lys Glu Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp 370 375 380	1152
TGG ATC GTC AGT GCG GAT TAT TCC CAA ATA GAA CTC AGA ATC CTC GCT Trp Ile Val Ser Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala 385 390 395 400	1200
CAT CTC AGT GGT GAT GAG AAC CTT GTG AAG GCC TTC GAG GAG GGC ATC His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile 405 410 415	1248
GAT GTG CAC ACC TTG ACT GCC TCC AGG ATC TAC AAC GTA AAG CCA GAA Asp Val His Thr Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu 420 425 430	1296
GAA GTG AAC GAA GAA ATG CGA CGG GTT GGA AAG ATG GTG AAC TTC TCT Glu Val Asn Glu Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser 435 440 445	1344
ATA ATA TAC GGT GTC ACA CCG TAC GGT CTT TCT GTG AGA CTT GGA ATA Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile 450 455 460	1392
CCG GTT AAA GAA GCA GAA AAG ATG ATT ATC AGC TAT TTC ACA CTG TAT Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr 465 470 475 480	1440

465	470	475	480	
CCA AAG GTG CGA AGC TAC ATC CAG CAG GTT GTT GCA GAG GCA AAA GAG				1488
Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu	485	490	495	
AAG GGC TAC GTC AGG ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG				1536
Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln	500	505	510	
CTC ATG GCA AGG GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA				1584
Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala	515	520	525	
ATA AAC ACC CCC ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT				1632
Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala	530	535	540	
ATG ATA GAT ATA GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA				1680
Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg	545	550	555	560
ATG ATC ATT CAG GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG				1728
Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu	565	570	575	
GAA AAA GAA GAA CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG				1776
Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val	580	585	590	
GTG AAA CTC TCT GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC				1824
Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser	595	600	605	
TGG TCT TG				1833
Trp Ser				
610				

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met	Lys	Glu	Leu	Gln	Leu	Tyr	Glu	Glu	Ala	Glu	Pro	Thr	Gly	Tyr	Glu
1				5					10					15	
Ile	Val	Lys	Asp	His	Lys	Thr	Phe	Glu	Asp	Leu	Ile	Glu	Lys	Leu	Lys
			20					25					30		
Glu	Val	Pro	Ser	Phe	Ala	Leu	Ala	Leu	Glu	Thr	Ser	Ser	Leu	Asp	Pro
		35				40					45				
Phe	Asn	Cys	Glu	Ile	Val	Gly	Ile	Ser	Val	Ser	Phe	Lys	Pro	Lys	Thr
50						55					60				
Ala	Tyr	Tyr	Ile	Pro	Leu	His	His	Arg	Asn	Ala	Gln	Asn	Leu	Asp	Glu

65						70						75						80
Thr	Leu	Val	Leu	Ser	Lys	Leu	Lys	Glu	Ile	Leu	Glu	Asp	Pro	Ser	Ser			
				85					90					95				
Lys	Ile	Val	Gly	Gln	Asn	Leu	Lys	Tyr	Asp	Tyr	Lys	Val	Leu	Met	Val			
			100				105				110							
Lys	Gly	Ile	Ser	Pro	Val	Tyr	Pro	His	Phe	Asp	Thr	Met	Ile	Ala	Ala			
		115			120			125										
Tyr	Leu	Leu	Glu	Pro	Asn	Glu	Lys	Lys	Phe	Asn	Leu	Glu	Asp	Leu	Ser			
		130				135			140									
Leu	Lys	Phe	Leu	Gly	Tyr	Lys	Met	Thr	Ser	Tyr	Gln	Glu	Leu	Met	Ser			
145					150					155					160			
Phe	Ser	Ser	Pro	Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp			
				165					170					175				
Lys	Ala	Ala	Asn	Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu			
			180				185				190							
Tyr	Lys	Ile	Leu	Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val			
		195				200			205									
Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu			
		210				215			220									
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu			
225					230					235					240			
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile			
			245				250				255							
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile			
			260				265				270							
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr			
		275				280			285									
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu			
		290				295			300									
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu			
305					310					315					320			
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr			
				325					330					335				
Gly	Arg	Ile	His	Ala	Ser	Phe	His	Gln	Thr	Gly	Thr	Ala	Thr	Gly	Arg			
			340				345				350							
Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln	Asn	Leu	Pro	Thr	Lys	Ser	Glu			
		355				360			365									
Glu	Gly	Lys	Glu	Ile	Arg	Lys	Ala	Ile	Val	Pro	Gln	Asp	Pro	Asp	Trp			
		370				375			380									
Trp	Ile	Val	Ser	Ala	Asp	Tyr	Ser	Gln	Ile	Glu	Leu	Arg	Ile	Leu	Ala			
385					390					395					400			

His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile
 405 410 415
 Asp Val His Thr Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu
 420 425 430
 Glu Val Asn Glu Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser
 435 440 445
 Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile
 450 455 460
 Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr
 465 470 475 480
 Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu
 485 490 495
 Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln
 500 505 510
 Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala
 515 520 525
 Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala
 530 535 540
 Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg
 545 550 555 560
 Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu
 565 570 575
 Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val
 580 585 590
 Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
 595 600 605
 Trp Ser
 610

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCTGAAGTAC GCGTACAAGG TTCTTATGG

29

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCACACAGGA AACAGCTATG AC

22

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1833 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GAC CTT GAA ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GCG TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Ala Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT	432
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
130 135 140	
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG	480
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	

145					150					155					160					
TTT	TCC	TCA	CCA	CTT	TTT	GGT	TTC	AGC	TTT	GCG	GAT	GTT	CCG	GTA	GAC	528				
Phe	Ser	Ser	Pro	Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp					
				165					170					175						
AAG	GCT	GCG	AAC	TAC	TCC	TGC	GAG	GAT	GCA	GAC	ATC	ACT	TAT	AGG	CTC	576				
Lys	Ala	Ala	Asn	Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu					
			180					185					190							
TAC	AAG	ATA	CTC	AGC	ATG	AAG	CTC	CAT	GAA	GCG	GAA	CTT	GAG	AAC	GTC	624				
Tyr	Lys	Ile	Leu	Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val					
		195					200					205								
TTC	TAC	AGG	ATA	GAG	ATG	CCG	CTT	GTG	AAC	GTT	CTT	GCA	CGC	ATG	GAA	672				
Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu					
	210					215					220									
TTG	AAC	GGG	GTG	TAT	GTG	GAC	ACA	GAA	TTC	CTG	AAA	AAG	CTC	TCG	GAG	720				
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu					
225					230					235					240					
GAG	TAC	GGC	AAA	AAG	CTC	GAG	GAA	CTG	GCC	GAA	AAA	ATC	TAC	CAG	ATA	768				
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile					
				245					250					255						
GCA	GGA	GAG	CCC	TTC	AAC	ATC	AAT	TCT	CCA	AAA	CAG	GTT	TCA	AAG	ATC	816				
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile					
			260					265					270							
CTT	TTT	GAG	AAG	CTG	GGA	ATA	AAA	CCC	CGT	GGA	AAA	ACG	ACA	AAA	ACA	864				
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr					
		275					280					285								
GGA	GCG	TAC	TCT	ACC	AGG	ATA	GAG	GTG	TTG	GAA	GAG	ATA	GCG	AAT	GAG	912				
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu					
	290					295					300									
CAC	GAG	ATA	GTA	CCC	CTC	ATT	CTC	GAG	TAC	AGA	AAG	ATC	CAG	AAA	CTG	960				
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu					
305					310					315					320					
AAA	TCG	ACC	TAC	ATA	GAC	ACC	CTT	CCG	AAA	CTT	GTG	AAC	CCG	AAA	ACC	1008				
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr					
				325				330					335							
GGA	AGA	ATT	CAT	GCA	TCT	TTC	CAC	CAG	ACG	GGT	ACC	GCC	ACT	GGC	AGG	1056				
Gly	Arg	Ile	His	Ala	Ser	Phe	His	Gln	Thr	Gly	Thr	Ala	Thr	Gly	Arg					
			340					345					350							
TTG	AGT	AGC	AGT	GAT	CCA	AAT	CTT	CAG	AAT	CTT	CCG	ACA	AAG	AGC						

CAT	CTC	AGT	GGT	GAT	GAG	AAC	CTT	GTG	AAG	GCC	TTC	GAG	GAG	GGC	ATC	1248
His	Leu	Ser	Gly	Asp	Glu	Asn	Leu	Val	Lys	Ala	Phe	Glu	Glu	Gly	Ile	
			405						410					415		
GAT	GTG	CAC	ACC	TTG	ACT	GCC	TCC	AGG	ATC	TAC	AAC	GTA	AAG	CCA	GAA	1296
Asp	Val	His	Thr	Leu	Thr	Ala	Ser	Arg	Ile	Tyr	Asn	Val	Lys	Pro	Glu	
			420					425					430			
GAA	GTG	AAC	GAA	GAA	ATG	CGA	CGG	GTT	GGA	AAG	ATG	GTG	AAC	TTC	TCT	1344
Glu	Val	Asn	Glu	Glu	Met	Arg	Arg	Val	Gly	Lys	Met	Val	Asn	Phe	Ser	
		435					440					445				
ATA	ATA	TAC	GGT	GTC	ACA	CCG	TAC	GGT	CTT	TCT	GTG	AGA	CTT	GGA	ATA	1392
Ile	Ile	Tyr	Gly	Val	Thr	Pro	Tyr	Gly	Leu	Ser	Val	Arg	Leu	Gly	Ile	
	450					455					460					
CCG	GTT	AAA	GAA	GCA	GAA	AAG	ATG	ATT	ATC	AGC	TAT	TTC	ACA	CTG	TAT	1440
Pro	Val	Lys	Glu	Ala	Glu	Lys	Met	Ile	Ile	Ser	Tyr	Phe	Thr	Leu	Tyr	
465					470					475					480	
CCA	AAG	GTG	CGA	AGC	TAC	ATC	CAG	CAG	GTT	GTT	GCA	GAG	GCA	AAA	GAG	1488
Pro	Lys	Val	Arg	Ser	Tyr	Ile	Gln	Gln	Val	Val	Ala	Glu	Ala	Lys	Glu	
				485					490					495		
AAG	GGC	TAC	GTC	AGG	ACT	CTC	TTT	GGA	AGA	AAA	AGA	GAT	ATT	CCC	CAG	1536
Lys	Gly	Tyr	Val	Arg	Thr	Leu	Phe	Gly	Arg	Lys	Arg	Asp	Ile	Pro	Gln	
			500					505					510			
CTC	ATG	GCA	AGG	GAC	AAG	AAC	ACC	CAG	TCC	GAA	GGC	GAA	AGA	ATC	GCA	1584
Leu	Met	Ala	Arg	Asp	Lys	Asn	Thr	Gln	Ser	Glu	Gly	Glu	Arg	Ile	Ala	
		515					520					525				
ATA	AAC	ACC	CCC	ATT	CAG	GGA	ACG	GCG	GCA	GAT	ATA	ATA	AAA	TTG	GCT	1632
Ile	Asn	Thr	Pro	Ile	Gln	Gly	Thr	Ala	Ala	Asp	Ile	Ile	Lys	Leu	Ala	
	530					535					540					
ATG	ATA	GAT	ATA	GAC	GAG	GAG	CTG	AGA	AAA	AGA	AAC	ATG	AAA	TCC	AGA	1680
Met	Ile	Asp	Ile	Asp	Glu	Glu	Leu	Arg	Lys	Arg	Asn	Met	Lys	Ser	Arg	
545					550					555					560	
ATG	ATC	ATT	CAG	GTT	CAT	GAC	GAA	CTG	GTC	TTC	GAG	GTT	CCC	GAT	GAG	1728
Met	Ile	Ile	Gln	Val	His	Asp	Glu	Leu	Val	Phe	Glu	Val	Pro	Asp	Glu	
				565					570					575		
GAA	AAA	GAA	GAA	CTA	GTT	GAT	CTG	GTG	AAG	AAC	AAA	ATG	ACA	AAT	GTG	1776
Glu	Lys	Glu	Glu	Leu	Val	Asp	Leu	Val	Lys	Asn	Lys	Met	Thr	Asn	Val	
			580					585					590			
GTG	AAA	CTC	TCT	GTG	CCT	CTT	GAG	GTT	GAC	ATA	AGC	ATC	GGA	AAA	AGC	1824
Val	Lys	Leu	Ser	Val	Pro	Leu	Glu	Val	Asp	Ile	Ser	Ile	Gly	Lys	Ser	
		595					600					605				
TGG	TCT	TG														

(2) INFORMATION FOR SEQ ID NO:33:

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu
1 5 10 15
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys
20 25 30
Glu Val Pro Ser Phe Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro
35 40 45
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr
50 55 60
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu
65 70 75 80
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser
85 90 95
Lys Ile Val Gly Gln Asn Leu Lys Tyr Ala Tyr Lys Val Leu Met Val
100 105 110
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala
115 120 125
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser
130 135 140
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser
145 150 155 160
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp
165 170 175
Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu
180 185 190
Tyr Lys Ile Leu Ser Met Lys Leu His Glu Ala Glu Leu Glu Asn Val
195 200 205
Phe Tyr Arg Ile Glu Met Pro Leu Val Asn Val Leu Ala Arg Met Glu
210 215 220
Leu Asn Gly Val Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu
225 230 235 240
Glu Tyr Gly Lys Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile
245 250 255
Ala Gly Glu Pro Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile
260 265 270
Leu Phe Glu Lys Leu Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr
275 280 285
Gly Ala Tyr Ser Thr Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu
290 295 300
His Glu Ile Val Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu

00043319.004000

305 310 315 320
Lys Ser Thr Tyr Ile Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr
 325 330 335
Gly Arg Ile His Ala Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg
 340 345 350
Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu
 355 360 365
Glu Gly Lys Glu Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp
 370 375 380
Trp Ile Val Ser Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala
385 390 395 400
His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile
 405 410 415
Asp Val His Thr Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu
 420 425 430
Glu Val Asn Glu Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser
 435 440 445
Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile
 450 455 460
Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr
465 470 475 480
Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu
 485 490 495
Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln
 500 505 510
Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala
 515 520 525
Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala
 530 535 540
Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg
545 550 555 560
Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu
 565 570 575
Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val
 580 585 590
Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
 595 600 605
Trp Ser
610

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1833 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATG AAG GAA CTT CAA CTG TAC GAA GAA GCA GAA CCC ACC GGA TAC GAA	48
Met Lys Glu Leu Gln Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu	
1 5 10 15	
ATC GTG AAG GAT CAT AAG ACC TTC GAA GAT CTC ATC GAA AAG CTG AAG	96
Ile Val Lys Asp His Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys	
20 25 30	
GAG GTT CCA TCT TTT GCC CTG GCC CTT GAA ACG TCC TCC CTT GAC CCG	144
Glu Val Pro Ser Phe Ala Leu Ala Leu Glu Thr Ser Ser Leu Asp Pro	
35 40 45	
TTC AAC TGT GAG ATA GTC GGC ATC TCC GTG TCG TTC AAA CCG AAA ACA	192
Phe Asn Cys Glu Ile Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr	
50 55 60	
GCT TAT TAC ATT CCA CTT CAT CAC AGA AAC GCC CAG AAT CTT GAT GAA	240
Ala Tyr Tyr Ile Pro Leu His His Arg Asn Ala Gln Asn Leu Asp Glu	
65 70 75 80	
ACA CTG GTG CTG TCG AAG TTG AAA GAG ATC CTC GAA GAC CCG TCT TCG	288
Thr Leu Val Leu Ser Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser	
85 90 95	
AAG ATT GTG GGT CAG AAC CTG AAG TAC GCG TAC AAG GTT CTT ATG GTA	336
Lys Ile Val Gly Gln Asn Leu Lys Tyr Ala Tyr Lys Val Leu Met Val	
100 105 110	
AAG GGT ATA TCG CCA GTT TAT CCG CAT TTT GAC ACG ATG ATA GCT GCA	384
Lys Gly Ile Ser Pro Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala	
115 120 125	
TAT TTG CTG GAG CCA AAC GAG AAA AAA TTC AAT CTC GAA GAT CTG TCT	432
Tyr Leu Leu Glu Pro Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser	
130 135 140	
TTG AAA TTT CTC GGA TAC AAA ATG ACG TCT TAT CAG GAA CTG ATG TCG	480
Leu Lys Phe Leu Gly Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser	
145 150 155 160	
TTT TCC TCA CCA CTT TTT GGT TTC AGC TTT GCG GAT GTT CCG GTA GAC	528
Phe Ser Ser Pro Leu Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp	
165 170 175	
AAG GCT GCG AAC TAC TCC TGC GAG GAT GCA GAC ATC ACT TAT AGG CTC	576
Lys Ala Ala Asn Tyr Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu	
180 185 190	
TAC AAG ATA CTC AGC ATG AAG CTC CAT GAA GCG GAA CTT GAG AAC GTC	624

ATA ATA TAC GGT GTC ACA CCG TAC GGT CTT TCT GTG AGA CTT GGA ATA Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile 450 455 460	1392
CCG GTT AAA GAA GCA GAA AAG ATG ATT ATC AGC TAT TTC ACA CTG TAT Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr 465 470 475 480	1440
CCA AAG GTG CGA AGC TAC ATC CAG CAG GTT GTT GCA GAG GCA AAA GAG Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu 485 490 495	1488
AAG GGC TAC GTC AGG ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln 500 505 510	1536
CTC ATG GCA AGG GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala 515 520 525	1584
ATA AAC ACC CCC ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala 530 535 540	1632
ATG ATA GAT ATA GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg 545 550 555 560	1680
ATG ATC ATT CAG GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu 565 570 575	1728
GAA AAA GAA GAA CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val 580 585 590	1776
GTG AAA CTC TCT GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser 595 600 605	1824
TGG TCT TG Trp Ser 610	1833

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met	Lys	Glu	Leu	Gln	Leu	Tyr	Glu	Glu	Ala	Glu	Pro	Thr	Gly	Tyr	Glu
1				5					10					15	
Ile	Val	Lys	Asp	His	Lys	Thr	Phe	Glu	Asp	Leu	Ile	Glu	Lys	Leu	Lys
			20					25					30		
Glu	Val	Pro	Ser	Phe	Ala	Leu	Ala	Leu	Glu	Thr	Ser	Ser	Leu	Asp	Pro

35	40	45																	
Phe	Asn	Cys	Glu	Ile	Val	Gly	Ile	Ser	Val	Ser	Phe	Lys	Pro	Lys	Thr				
50						55					60								
Ala	Tyr	Tyr	Ile	Pro	Leu	His	His	Arg	Asn	Ala	Gln	Asn	Leu	Asp	Glu				
65					70					75					80				
Thr	Leu	Val	Leu	Ser	Lys	Leu	Lys	Glu	Ile	Leu	Glu	Asp	Pro	Ser	Ser				
				85					90					95					
Lys	Ile	Val	Gly	Gln	Asn	Leu	Lys	Tyr	Ala	Tyr	Lys	Val	Leu	Met	Val				
			100					105					110						
Lys	Gly	Ile	Ser	Pro	Val	Tyr	Pro	His	Phe	Asp	Thr	Met	Ile	Ala	Ala				
		115					120					125							
Tyr	Leu	Leu	Glu	Pro	Asn	Glu	Lys	Lys	Phe	Asn	Leu	Glu	Asp	Leu	Ser				
	130					135					140								
Leu	Lys	Phe	Leu	Gly	Tyr	Lys	Met	Thr	Ser	Tyr	Gln	Glu	Leu	Met	Ser				
145					150					155					160				
Phe	Ser	Ser	Pro	Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp				
				165					170					175					
Lys	Ala	Ala	Asn	Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu				
			180					185					190						
Tyr	Lys	Ile	Leu	Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val				
		195					200					205							
Phe	Tyr	Arg	Ile	Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu				
	210					215					220								
Leu	Asn	Gly	Val	Tyr	Val	Asp	Thr	Glu	Phe	Leu	Lys	Lys	Leu	Ser	Glu				
225					230					235					240				
Glu	Tyr	Gly	Lys	Lys	Leu	Glu	Glu	Leu	Ala	Glu	Lys	Ile	Tyr	Gln	Ile				
			245						250					255					
Ala	Gly	Glu	Pro	Phe	Asn	Ile	Asn	Ser	Pro	Lys	Gln	Val	Ser	Lys	Ile				
			260					265					270						
Leu	Phe	Glu	Lys	Leu	Gly	Ile	Lys	Pro	Arg	Gly	Lys	Thr	Thr	Lys	Thr				
		275					280					285							
Gly	Ala	Tyr	Ser	Thr	Arg	Ile	Glu	Val	Leu	Glu	Glu	Ile	Ala	Asn	Glu				
	290					295					300								
His	Glu	Ile	Val	Pro	Leu	Ile	Leu	Glu	Tyr	Arg	Lys	Ile	Gln	Lys	Leu				
305					310					315					320				
Lys	Ser	Thr	Tyr	Ile	Asp	Thr	Leu	Pro	Lys	Leu	Val	Asn	Pro	Lys	Thr				
				325					330					335					
Gly	Arg	Ile	His	Ala	Ser	Phe	His	Gln	Thr	Gly	Thr	Ala	Thr	Gly	Arg				
			340					345					350						
Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln	Asn	Leu	Pro	Thr	Lys	Ser	Glu				
			355				360					365							

Glu Gly Lys Glu Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp
 370 375 380
 Trp Ile Val Ser Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala
 385 390 395 400
 His Leu Ser Gly Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile
 405 410 415
 Asp Val His Thr Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu
 420 425 430
 Glu Val Asn Glu Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser
 435 440 445
 Ile Ile Tyr Gly Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile
 450 455 460
 Pro Val Lys Glu Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr
 465 470 475 480
 Pro Lys Val Arg Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu
 485 490 495
 Lys Gly Tyr Val Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln
 500 505 510
 Leu Met Ala Arg Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala
 515 520 525
 Ile Asn Thr Pro Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala
 530 535 540
 Met Ile Asp Ile Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg
 545 550 555 560
 Met Ile Ile Gln Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu
 565 570 575
 Glu Lys Glu Glu Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val
 580 585 590
 Val Lys Leu Ser Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser
 595 600 605
 Trp Ser
 610

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1716 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1716

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATG CTT GAA ACG TCC TCC CTT GAC CCG TTC AAC TGT GAG ATA GTC GGC	48
Met Leu Glu Thr Ser Ser Leu Asp Pro Phe Asn Cys Glu Ile Val Gly	
1 5 10 15	
ATC TCC GTG TCG TTC AAA CCG AAA ACA GCT TAT TAC ATT CCA CTT CAT	96
Ile Ser Val Ser Phe Lys Pro Lys Thr Ala Tyr Tyr Ile Pro Leu His	
20 25 30	
CAC AGA AAC GCC CAG AAT CTT GAT GAA ACA CTG GTG CTG TCG AAG TTG	144
His Arg Asn Ala Gln Asn Leu Asp Glu Thr Leu Val Leu Ser Lys Leu	
35 40 45	
AAA GAG ATC CTC GAA GAC CCG TCT TCG AAG ATT GTG GGT CAG AAC CTG	192
Lys Glu Ile Leu Glu Asp Pro Ser Ser Lys Ile Val Gly Gln Asn Leu	
50 55 60	
AAG TAC GAC TAC AAG GTT CTT ATG GTA AAG GGT ATA TCG CCA GTT TAT	240
Lys Tyr Asp Tyr Lys Val Leu Met Val Lys Gly Ile Ser Pro Val Tyr	
65 70 75 80	
CCG CAT TTT GAC ACG ATG ATA GCT GCA TAT TTG CTG GAG CCA AAC GAG	288
Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro Asn Glu	
85 90 95	
AAA AAA TTC AAT CTC GAA GAT CTG TCT TTG AAA TTT CTC GGA TAC AAA	336
Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly Tyr Lys	
100 105 110	
ATG ACG TCT TAT CAG GAA CTG ATG TCG TTT TCC TCA CCA CTT TTT GGT	384
Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Ser Pro Leu Phe Gly	
115 120 125	
TTC AGC TTT GCG GAT GTT CCG GTA GAC AAG GCT GCG AAC TAC TCC TGC	432
Phe Ser Phe Ala Asp Val Pro Val Asp Lys Ala Ala Asn Tyr Ser Cys	
130 135 140	
GAG GAT GCA GAC ATC ACT TAT AGG CTC TAC AAG ATA CTC AGC ATG AAG	480
Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Ile Leu Ser Met Lys	
145 150 155 160	
CTC CAT GAA GCG GAA CTT GAG AAC GTC TTC TAC AGG ATA GAG ATG CCG	528
Leu His Glu Ala Glu Leu Glu Asn Val Phe Tyr Arg Ile Glu Met Pro	
165 170 175	
CTT GTG AAC GTT CTT GCA CGC ATG GAA TTG AAC GGG GTG TAT GTG GAC	576
Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr Val Asp	
180 185 190	
ACA GAA TTC CTG AAA AAG CTC TCG GAG GAG TAC GGC AAA AAG CTC GAG	624
Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys Leu Glu	
195 200 205	
GAA CTG GCC GAA AAA ATC TAC CAG ATA GCA GGA GAG CCC TTC AAC ATC	672
Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe Asn Ile	
210 215 220	
AAT TCT CCA AAA CAG GTT TCA AAG ATC CTT TTT GAG AAG CTG GGA ATA	720
Asn Ser Pro Lys Gln Val Ser Lys Ile Leu Phe Glu Lys Leu Gly Ile	
225 230 235 240	

485								490				495				
ACG	GCG	GCA	GAT	ATA	ATA	AAA	TTG	GCT	ATG	ATA	GAT	ATA	GAC	GAG	GAG	1536
Thr	Ala	Ala	Asp	Ile	Ile	Lys	Leu	Ala	Met	Ile	Asp	Ile	Asp	Glu	Glu	
500								505				510				
CTG	AGA	AAA	AGA	AAC	ATG	AAA	TCC	AGA	ATG	ATC	ATT	CAG	GTT	CAT	GAC	1584
Leu	Arg	Lys	Arg	Asn	Met	Lys	Ser	Arg	Met	Ile	Ile	Gln	Val	His	Asp	
515								520				525				
GAA	CTG	GTC	TTC	GAG	GTT	CCC	GAT	GAG	GAA	AAA	GAA	GAA	CTA	GTT	GAT	1632
Glu	Leu	Val	Phe	Glu	Val	Pro	Asp	Glu	Glu	Lys	Glu	Glu	Leu	Val	Asp	
530								535				540				
CTG	GTG	AAG	AAC	AAA	ATG	ACA	AAT	GTG	GTG	AAA	CTC	TCT	GTG	CCT	CTT	1680
Leu	Val	Lys	Asn	Lys	Met	Thr	Asn	Val	Val	Lys	Leu	Ser	Val	Pro	Leu	
545								550				555				
GAG	GTT	GAC	ATA	AGC	ATC	GGA	AAA	AGC	TGG	TCT	TG					1716
Glu	Val	Asp	Ile	Ser	Ile	Gly	Lys	Ser	Trp	Ser						
565								570								

(2) INFORMATION FOR SEQ ID NO:37:

(ii) MOLECULE TYPE: protein

Met 1	Leu	Glu	Thr	Ser 5	Ser	Leu	Asp	Pro	Phe 10	Asn	Cys	Glu	Ile	Val 15	Gly
Ile	Ser	Val	Ser 20	Phe	Lys	Pro	Lys	Thr 25	Ala	Tyr	Tyr	Ile	Pro 30	Leu	His
His	Arg	Asn 35	Ala	Gln	Asn	Leu	Asp 40	Glu	Thr	Leu	Val	Leu 45	Ser	Lys	Leu
Lys	Glu 50	Ile	Leu	Glu	Asp	Pro 55	Ser	Ser	Lys	Ile	Val 60	Gly	Gln	Asn	Leu
Lys 65	Tyr	Asp	Tyr	Lys	Val 70	Leu	Met	Val	Lys	Gly 75	Ile	Ser	Pro	Val	Tyr 80
Pro	His	Phe	Asp	Thr 85	Met	Ile	Ala	Ala	Tyr 90	Leu	Leu	Glu	Pro	Asn 95	Glu
Lys	Lys	Phe	Asn 100	Leu	Glu	Asp	Leu	Ser 105	Leu	Lys	Phe	Leu	Gly 110	Tyr	Lys
Met	Thr	Ser 115	Tyr	Gln	Glu	Leu	Met 120	Ser	Phe	Ser	Ser	Pro 125	Leu	Phe	Gly
Phe	Ser 130	Phe	Ala	Asp	Val	Pro 135	Val	Asp	Lys	Ala	Ala 140	Asn	Tyr	Ser	Cys
Glu 145	Asp	Ala	Asp	Ile	Thr 150	Tyr	Arg	Leu	Tyr	Lys 155	Ile	Leu	Ser	Met	Lys 160

Leu His Glu Ala Glu Leu Glu Asn Val Phe Tyr Arg Ile Glu Met Pro
 165 170 175
 Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr Val Asp
 180 185 190
 Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys Leu Glu
 195 200 205
 Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe Asn Ile
 210 215 220
 Asn Ser Pro Lys Gln Val Ser Lys Ile Leu Phe Glu Lys Leu Gly Ile
 225 230 235 240
 Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Ala Tyr Ser Thr Arg Ile
 245 250 255
 Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val Pro Leu Ile
 260 265 270
 Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr Ile Asp Thr
 275 280 285
 Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Ile His Ala Ser Phe
 290 295 300
 His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp Pro Asn
 305 310 315 320
 Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile Arg Lys
 325 330 335
 Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser Ala Asp Tyr
 340 345 350
 Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp Glu Asn
 355 360 365
 Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu Thr Ala
 370 375 380
 Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu Glu Met Arg
 385 390 395 400
 Arg Val Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly Val Thr Pro
 405 410 415
 Tyr Gly Leu Ser Val Arg Leu Gly Ile Pro Val Lys Glu Ala Glu Lys
 420 425 430
 Met Ile Ile Ser Tyr Phe Thr Leu Tyr Pro Lys Val Arg Ser Tyr Ile
 435 440 445
 Gln Gln Val Val Ala Glu Ala Lys Glu Lys Gly Tyr Val Arg Thr Leu
 450 455 460
 Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg Asp Lys Asn
 465 470 475 480
 Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro Ile Gln Gly
 485 490 495

[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (ii) MOLECULE TYPE: DNA (genomic)

(A) NAME/KEY: CDS

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

- 171 -

370	375	380	
AGG ACT CTC TTT GGA AGA AAA AGA GAT ATT CCC CAG CTC ATG GCA AGG			1200
Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg			
385	390	395	400
GAC AAG AAC ACC CAG TCC GAA GGC GAA AGA ATC GCA ATA AAC ACC CCC			1248
Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro			
	405	410	415
ATT CAG GGA ACG GCG GCA GAT ATA ATA AAA TTG GCT ATG ATA GAT ATA			1296
Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Asp Ile			
	420	425	430
GAC GAG GAG CTG AGA AAA AGA AAC ATG AAA TCC AGA ATG ATC ATT CAG			1344
Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg Met Ile Ile Gln			
	435	440	445
GTT CAT GAC GAA CTG GTC TTC GAG GTT CCC GAT GAG GAA AAA GAA GAA			1392
Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu Glu Lys Glu Glu			
	450	455	460
CTA GTT GAT CTG GTG AAG AAC AAA ATG ACA AAT GTG GTG AAA CTC TCT			1440
Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val Val Lys Leu Ser			
	465	470	475
GTG CCT CTT GAG GTT GAC ATA AGC ATC GGA AAA AGC TGG TCT TG			1485
Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser Trp Ser			
	485	490	495

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 494 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met	Lys	Glu	Leu	Gln	Leu	Tyr	Glu	Glu	Ala	Glu	Pro	Thr	Gly	Tyr	Glu
1				5					10					15	
Ile	Val	Lys	Asp	His	Lys	Thr	Phe	Glu	Asp	Leu	Ser	Leu	Lys	Phe	Leu
		20					25						30		
Gly	Tyr	Lys	Met	Thr	Ser	Tyr	Gln	Glu	Leu	Met	Ser	Phe	Ser	Ser	Pro
		35					40					45			
Leu	Phe	Gly	Phe	Ser	Phe	Ala	Asp	Val	Pro	Val	Asp	Lys	Ala	Ala	Asn
		50				55					60				
Tyr	Ser	Cys	Glu	Asp	Ala	Asp	Ile	Thr	Tyr	Arg	Leu	Tyr	Lys	Ile	Leu
		65			70				75					80	
Ser	Met	Lys	Leu	His	Glu	Ala	Glu	Leu	Glu	Asn	Val	Phe	Tyr	Arg	Ile
			85					90						95	
Glu	Met	Pro	Leu	Val	Asn	Val	Leu	Ala	Arg	Met	Glu	Leu	Asn	Gly	Val
			100				105						110		

09644349-081300

Tyr Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys
115 120 125

Lys Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro
130 135 140

Phe Asn Ile Asn Ser Pro Lys Gln Val Ser Lys Ile Leu Phe Glu Lys
145 150 155 160

Leu Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Ala Tyr Ser
165 170 175

Thr Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val
180 185 190

Pro Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr
195 200 205

Ile Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Ile His
210 215 220

Ala Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser
225 230 235 240

Asp Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu
245 250 255

Ile Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser
260 265 270

Ala Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly
275 280 285

Asp Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr
290 295 300

Leu Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu
305 310 315 320

Glu Met Arg Arg Val Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly
325 330 335

Val Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile Pro Val Lys Glu
340 345 350

Ala Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr Pro Lys Val Arg
355 360 365

Ser Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu Lys Gly Tyr Val
370 375 380

Arg Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg
385 390 395 400

Asp Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro
405 410 415

Ile Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Asp Ile
420 425 430

Asp Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg Met Ile Ile Gln
435 440 445

Val His Asp Glu Leu Val Phe Glu Val Pro Asp Glu Glu Lys Glu Glu
 450 455 460

Leu Val Asp Leu Val Lys Asn Lys Met Thr Asn Val Val Lys Leu Ser
 465 470 475 480

Val Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser Trp Ser
 485 490

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CGCCAGGGTT TTCCAGTCA CGAC

24

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 74 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATAAGCGCCA TTGATGTTCC TCTCTACTCG AAAGTTAGAG AGGACACACC CGATCCCTAT
 AGTGAGTCGT ATTA

60

74

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TAATACGACT CACTATAGGG CGAAT

25

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

[illegible]

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

33

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

29

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

25

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CATGCCATGG CATGCATTTA CGTTGACACC A

31

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TCCCCCGGGT TGCGCTCACT GCCCGCTTTC CAGT

34

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AGCTTATCGA TGGCACTTTT CGGGGAAATG TGCG

34

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AGCTTATCGA TAAGCGATGC CGGGAGCAGA CAAGC

35

CLAIMS

1. A purified thermostable DNA polymerase capable of DNA synthetic activity, said enzyme derived from the eubacterium *Thermotoga neapolitana*.

2. The polymerase of Claim 1, wherein said polymerase is further capable of 3' exonuclease activity.

3. The polymerase of Claim 1, wherein said polymerase is further capable of 5' exonuclease activity.

4. The polymerase of Claim 1, wherein said polymerase comprises the amino acid sequence of SEQ ID NO:2.

5. The polymerase of Claim 1, wherein the specific activity of said synthetic activity is approximately 100,000 units/mg.

6. A purified thermostable non-naturally occurring DNA polymerase derived from the eubacterium *Thermotoga neapolitana*.

7. The polymerase of Claim 6, wherein said polymerase is capable of 3' exonuclease activity.

8. The polymerase of Claim 6, wherein said polymerase is capable of 5' exonuclease activity.

9. The polymerase of Claim 6, wherein said polymerase has reduced levels of 3' exonuclease activity.

10. The polymerase of Claim 6, wherein said polymerase lacks significant 5' exonuclease activity.

11. The polymerase of Claim 6, wherein said polymerase comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:8, 16, 19, 23, 26, 29, 33 and 35.

12. An oligonucleotide comprising the nucleic acid sequence of SEQ ID NO:1, said nucleotide sequence encoding a thermostable DNA polymerase.

13. The oligonucleotide of Claim 12, wherein said sequence is modified such that the encoded polymerase lacks significant 5' exonuclease activity.

14. The oligonucleotide of Claim 13, wherein said modified sequence comprises the nucleotide sequence of SEQ ID NO:7.

15. The oligonucleotide of Claim 12, wherein said sequence is modified such that the encoded polymerase displays reduced levels of 3' exonuclease activity.

16. The oligonucleotide of Claim 15, wherein said modified sequence is selected from the group consisting of SEQ ID NOS:7, 15, 18, 22, 25, 28, 32 and 34.

17. A recombinant DNA vector comprising an oligonucleotide having the nucleic acid sequence set forth in SEQ ID NO:1, said nucleotide sequence encoding a thermostable DNA polymerase having DNA synthetic activity.

18. The recombinant DNA vector of Claim 17, wherein said nucleic acid sequence is modified such that the encoded thermostable DNA polymerase lacks significant 5' exonuclease activity.

19. The recombinant vector of Claim 18, wherein said modified sequence comprises SEQ ID NO:7.

20. The recombinant DNA vector of Claim 17, wherein said nucleic acid sequence is modified such that the encoded thermostable DNA polymerase exhibits reduced levels 3' exonuclease activity.

21. The recombinant vector of Claim 20, wherein said modified sequence is selected from the group consisting of SEQ ID NOS:7, 15, 18, 22, 25, 28, 32 and 34.

ABSTRACT

The present invention relates to thermostable DNA polymerases derived from the hyperthermophilic eubacteria, and *Thermotoga neapolitana* in particular. The present invention provides means for isolating and producing the enzymes from these thermostable DNA polymerases, which are useful in many recombinant DNA techniques, especially such techniques as thermal cycle sequencing and nucleic acid amplification.

5

09641319-081300

FIGURE 1

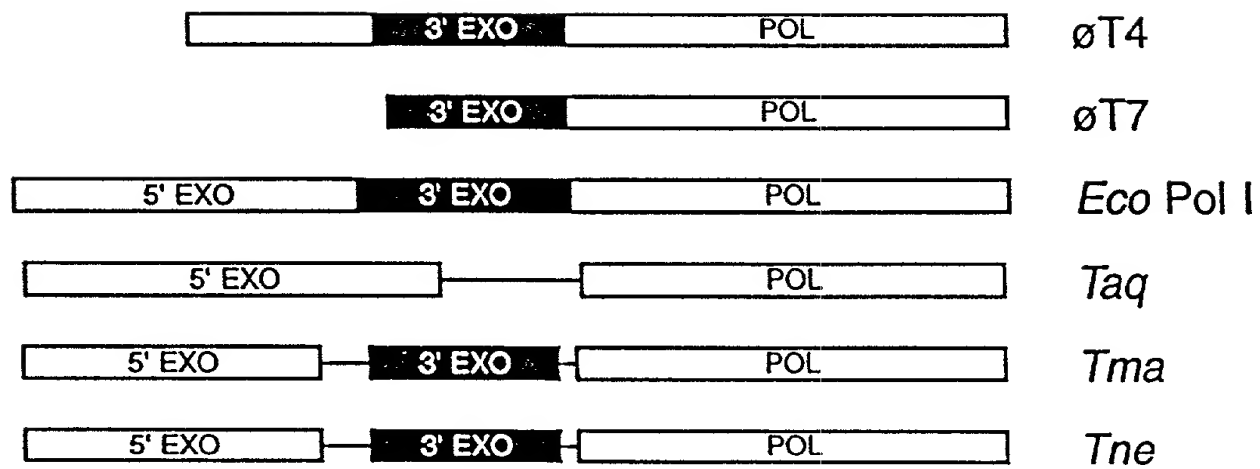


FIGURE 2

	Exo I			Exo II			Exo III		
			Δ Δ ∇	∇	Δ		Δ Δ Δ		
Bsu Pol III	419	ETYV	VFDVETTGLSAVY		LVAHN-A-SFDMGFNL	502	TLCKKE	-DIELTQH	552
Eco Pol III ϵ	6	TRQL	VLDNETTGMNQIG		LVIHN-AA-FDIGFMD	95	ALCARV	-EIDNSKR	147
ϕ T4	182	RVIYMP	FDNERDMLMEYI		FTGWNII-EGFDVPYIM	210	DKIRGF	--IDLVL	332
ϕ T7	1	---	MIVSDHEANALLESV		V-FHNGH-KYDVPALT	57	EEMMDV	NVQDVVVT	165
Eco Pol I	348	KAPV	FAFDNETDSLDNIS		V-GQNL--KYDRGILA	417	EEAGRV	AAEDADVT	492
Tma	316	ESPSFA	IDLETSSLDPPFD		V-GQNL--KFDYKVIM	382	EKAANY	SCEDADIT	459
Tne	316	EVPSFA	IDLETSSLDPPFN		V-GQNL--KYDYKVIM	382	DKAANY	SCEDADIT	459

FIGURE 3

1 MVOIPQNPILVDCSSYLVRAYHAF-PPLTNSAGEPTICAMYGVLMRLSLIMOY---KPTHAAVWFDKCKTFRDELFEH Eco
 1 MAR-----LFLFDGTALAYRAYYALDRSLSTSTGIPINATYGVARMLVRFIKDHIIVGKDYVAVAFDKKAATFRHKLLEI Tma
 1 MAR-----LFLFDGTALAYRAYYALDRSLSTSTGIPINAVYGVARMLVKFIKEHIIPEKDYA AVAFDKKAATFRHKLLEA The

 77 YKSHRPMPDILRAQIEPLHAMVKAMGLPLIAVSGVEADDVIGTLAREAEKAGRPVLI STGDKDMAQLVTPNITL---IN Eco
 76 YKAQRPKTPDLLIQQLPYIKKLVLEALGMKVLEVEGYEADDI IATLAVKGLPLIFDEIFIVTGDKDMLQLVNEKIKVWRIVK Tma
 76 YKAQRPKTPDLLVQQLPYIKRLIEALGFKVLELEGYEADDI IATLAVKGCTFFDEIFITGDKDMLQLVNEKIKVWRIVK The

 154 TWNTNII-GPEEVNKKYGVPEPIIIDEFLALMGDSSDNIPGVPGVGEKTAQALL OGLGGLDTLYAEPEKLAGLSERCAKTM Eco
 156 GISDLELYDAQKVKEKYGVPEQOIPDLLALTGDEIDNIPGVGTGIGEKTA VQLLEKYKLEDII---NHVRELPOKVRKA- Tma
 156 GISDLELYDSKKVKERYGVPEHQIPDLLALTGDEIDNIPGVGTGIGEKTA VQLLGKYRNLEDII---EHARELPQVRKA- The

 233 AAKLEONKEVAVLSYOLATIKIDVELELTCEOLEVOOPAAEELI GLEKKYEFKRWTADVEAGKWLQAKGAKPAKPOETS Eco
 232 ---LLRDRENAILSKKLAILLETNVPHEINWHEELRYOGYDREKLLPLKELEF-----ASIMKELO Tma
 232 ---LLRDRENAILSKKLATLVINAPVEVDWEEMKYRGYDKRKLPLKELEF-----ASIMKELO The

 313 VADEAPEVTATVISYDNYVTILDEETLKAWIAKLEKAPVFAFDIETISLDNISANIVGISEATEPGVAAYIRVAHDYILDA Eco
 289 LYEESEPVG-----YRIVKDLVEFEKLEIEKRESPSFAIDLETSSLDPFDDIVGISVSFKPKKAYYIPLHHR---N Tma
 289 LYEEAEPTG-----YEIVKDHKTFEDLIEKLEKVPSPFALDLETSSLDPFNCEIVGISVSFKPKKTAYYIPLHHR---N The

 393 PDOISRERALEILKPLLEDEKALKVGNLKYDRGILANYGIELRGIAFDIMLESYILNSVAGRHDMDSLAERALKHKTTT Eco
 358 AONLDEKEVLEKLEKEILEDEPKAKIVGNLKEIDYKVLVKGVEPVPPYFDIMIAAYLLEPNEKKFNLDLALKFLGYKMTS Tma
 358 AONLDETLLVLSKLEKEILEDPSSKIVGNLKYDYKVLVKGISPVYPHFDMIAAYLLEPNEKKFNLEDLSLKFLGYKMTS The

 473 FEETAGKGN--OLTFNOTALEEAGRYAAEDADVILOLHLKMPDLOKHKGPL-NVFENIEMPLVEVLSEIERNGVKIDP Eco
 438 YOELMSFSFPLFGFSFADVPVEKAANYSCEDADITYRLYKII--SUKLHEADLENVFKIEMPLVNVLARMELNGVYVDT Tma
 438 YOELMSFSFPLFGFSFADVPVDKAANYSCEDADITYRLYKII--SMKLHEAELENVFYRIEMPLVNVLARMELNGVYVDT The

 550 KVLHNESEELTTRIAELERKAHEIAGEEFNLSSIKOLOTLFEKOGIKPL-KKTPGGAPSTSEVLEEIALDYPLPKVIL Eco
 516 EFLKKLSEEYGKKLEELAEETIYRIAGEPFNINSPKQVSRILFEKLGKPRGKTITKTGYSTRIEVLEEIAGEHEIIPILIL Tma
 516 EFLKKLSEEYGKKLEELAEKIYQIAGEPFNINSPKQVSKILFEKLGKPRGKTITKTGAYSTRIEVLEEIANEHEITVPLIL The

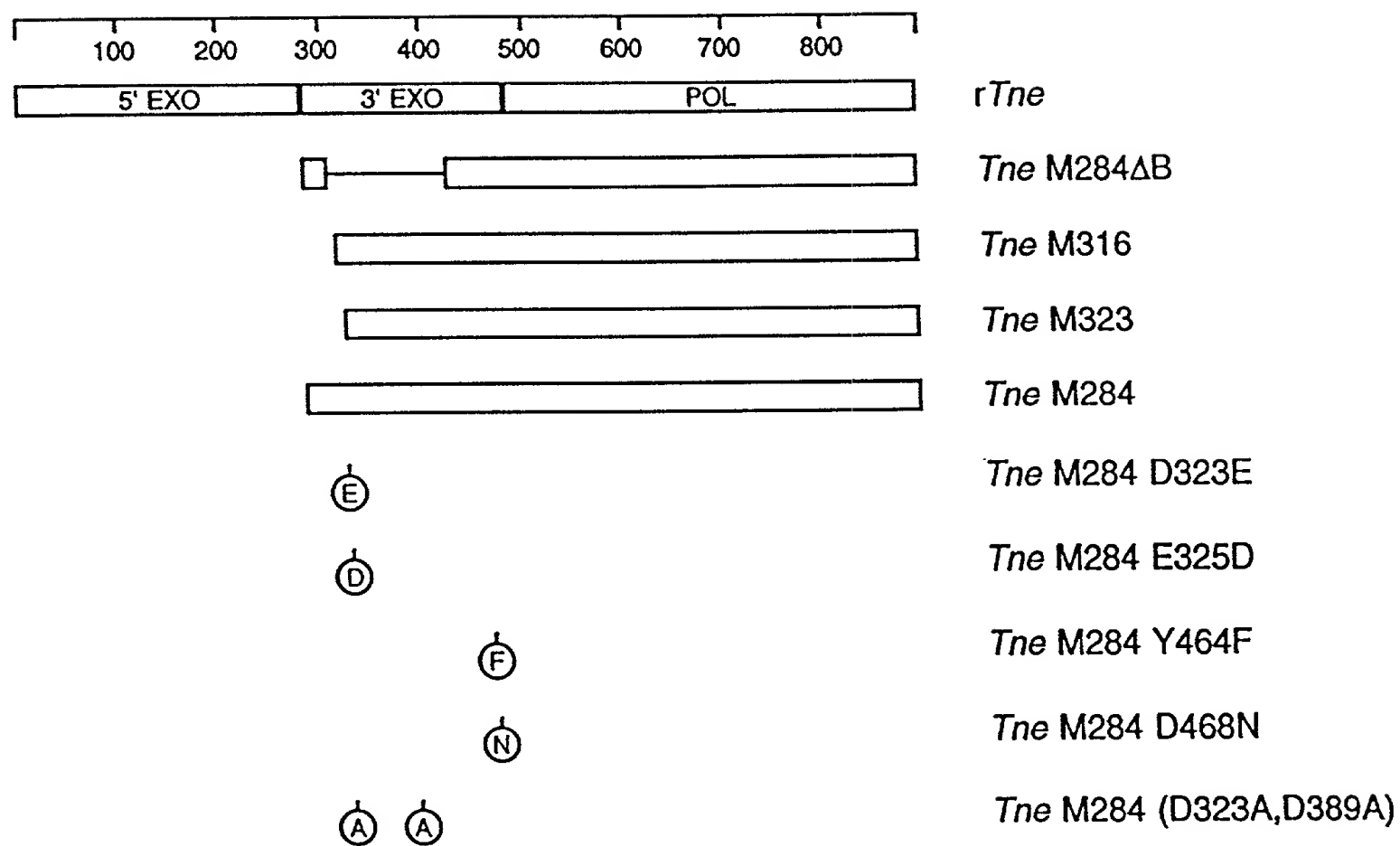
 629 EYRGLAKLKSTYIDKLPIMINPKTGRIHAFSYHOAVTATGRLSSIDPNLQNIIVRNEEGRRIRCAFTAPE-DYVIVSADYS Eco
 596 EYRKIQKLKSTYIDALPKMVNPKTGRIHASFNQGTATGRLSSSDPNLQNLPTKSEEGKEIRKAIVPQDPNWWIVSADYS Tma
 596 EYRKIQKLKSTYIDTLPKLVNPKTGRIHASFHQGTATGRLSSSDPNLQNLPTKSEEGKEIRKAIVPQDPDWWIVSADYS The

 708 QIELRIMAHLSRDKCULTAFABECKDIHRATAAEVFGLEPLEITVISEORRSKATINFGIYGMFAFGIAROLNIPRKEACKY Eco
 676 QIELRILAHLSGDENLIRAFEEGIDVHTLTASRIENVKPEEVIEEMRRAGKVMNFSSIIYGVTPYGLSVRLGVPVKEAEKM Tma
 676 QIELRILAHLSGDENLVKAFEEGIDVHTLTASRIYNVKPEEVNEEMRRVKGVMNFSSIIYGVTPYGLSVRLGIPVKEAEKM The

 788 MDLYFERYPGVLEYMERTRAOAKEOGYVETLDGRRLYLPDIKSSNGARRAAAERAANAPMOGTAADI IKRAMIAVDAML Eco
 756 IVNYFVLYPKVRDYIQRVVS EAKEKGYVRTLFGRKRDIPQLMARDKNTQAEGERIAINTPIQGTAAADI IKLAMIEIDREL Tma
 756 IISYFTLYPKVRSYIQQVVAEAKEKGYVRTLFGRKRDIPQLMARDKNTQSEGERIAINTPIQGTAAADI IKLAMIDIDEEL The

 868 QAEQPRVRMIMQVHDELVFEVHKDDVDVAKQIHQUMENCTRLDVPLIVEVSGSENWDQAH. Eco
 836 KERKMRSMIIQVHDELVFEVPNEEKDALVPLVKDRMTNVVKSVPLEVDVIGKTS. Tma
 836 RKRNMKSRMIIQVHDELVFEVPDEEKEELVDLVKNKMTNVVKSVPLEVDISIGKSW. The

FIGURE 4



0964139-034300

[illegible]

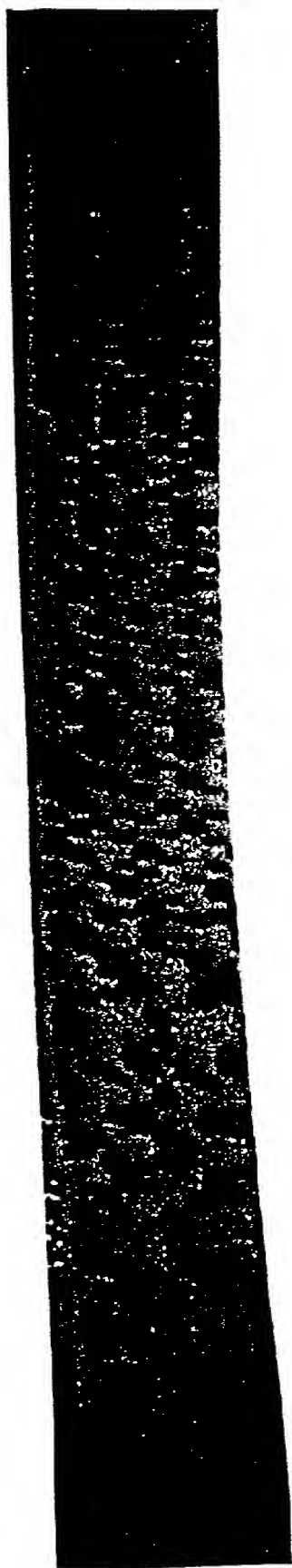
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1 2 3 4 5 6 7 8

FIGURE 6

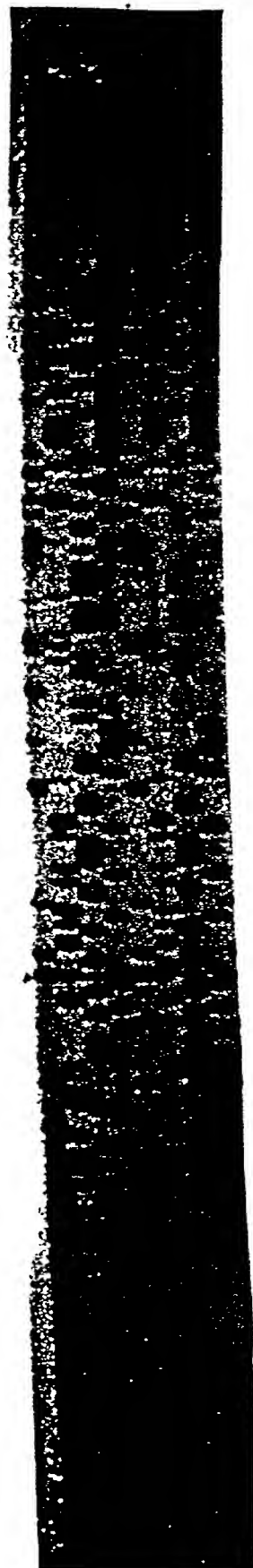
A

1 2 3 4 5 6 7 8



B

1 2 3 4 5 6 7 8



C

1 2 3 4 5 6 7 8



09644319-001000

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **THERMOPHILIC DNA POLYMERASES FROM THERMOTOGA NEAPOLITANA**, the specification of which was filed on 06/07/95 as Application Serial No. 08/484,661. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: Michael R. Slater

Inventor's Signature: Michael R. Slater

Date: 9 Sept. 1996

Residence: 118 North Breese Terrace, Madison, Wisconsin 53705

Citizenship: United States Of America

Post Office Address: 118 North Breese Terrace, Madison, Wisconsin 53705

Full Name of Second Joint Inventor: Fen Huang

Inventor's Signature: Fen Huang

Date: 9 / 9 / 1996

Residence: 1329 North Wingra Drive, Madison, Wisconsin 53715

Citizenship: United States Of America

Post Office Address: 1329 North Wingra Drive, Madison, Wisconsin 53715

Full Name of Third Joint Inventor: James R. Hartnett

Inventor's Signature: James R. Hartnett

Date: 9-9-96

Residence: 2590 Chesapeake Drive, Fitchburg, Wisconsin 53719

Citizenship: United States Of America

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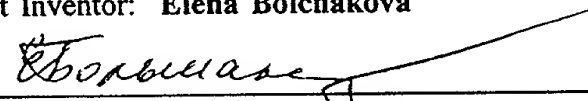
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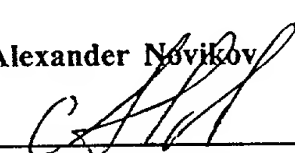
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Full Name of Fourth Joint Inventor: **Elena Bolchakova**

Inventor's Signature: 
Residence: 613 Catamaran, #1, Foster City, California 94404
Post Office Address: 613 Catamaran, #1, Foster City, California 94404

Date: 06.26.96
Citizenship: Russia

Full Name of Fifth Joint Inventor: **Alexander Novikov**

Inventor's Signature: 
Residence: 613 Catamaran, #1, Foster City, California 94404
Post Office Address: 613 Catamaran, #1, Foster City, California 94404

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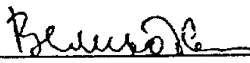
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Full Name of Sixth Joint Inventor: Galina A. Velikodvorskaya

Inventor's Signature: 
Residence: Lytovsky Blv., 13/12, Apt. 358, Moscow, Russia 117593
Post Office Address: Lytovsky Blv., 13/12, Apt. 358, Moscow, Russia 117593

Date: 05.08.1996
Citizenship: Russia

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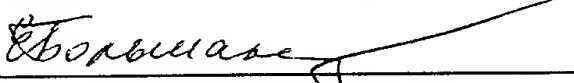
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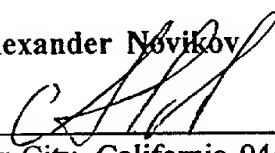
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Inventor's Signature: 

Date: 05.08.1996

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